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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: GNCA-P01-005

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/936035

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/27907	24 November 1999 (24.11.99)	25 November 1998 (25.11.98)

TITLE OF INVENTION: METHODS AND REAGENTS FOR INCREASING PROLIFERATIVE CAPACITY AND PREVENTING REPLICATIVE SENESCENCE

APPLICANT(S) FOR DO/EO/US: HANNON, Gregory J.; BEACH, David H.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C 371(c)(2)).
   a. [ ] is attached hereto.
   b. [X] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **Unexecuted**
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3 31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S C. 1.821 - 1 825.
18. [X] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: **Abstract (1 p.); Petition for Revival of An International Application ... (2 pp., dupl.); Certificate of Express Mailing (1 p.); postpaid return postcard**

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/936035	PCT/US99/27907	GNCA-P01-005

	CALCULATIONS PTO USE ONLY	
21. ☐ The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 710	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	143 - 20 =	123	x $18.00	$ 2,214	
Independent claims	9 - 3 =	6	x $80.00	$ 480	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 270	
TOTAL OF ABOVE CALCULATIONS =				$ 3,674	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$ 1,837	
SUBTOTAL =				$ 1,837	
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TOTAL NATIONAL FEE =				$ 1,837	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 1,837	
				Amount to be refunded:	$
				charged:	$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 18-1945 in the amount of $ 1,837.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1945 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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Ropes & Gray
One International Place
Boston, MA 02110
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Matthew P. Vincent
NAME

Reg. 36,709
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September 7, 2001

11/prts

Methods and Reagents for Increasing Proliferative Capacity and Preventing Replicative Senescence

Background of the Invention

Normal mammalian diploid cells placed in culture have a finite proliferative life-span and enter a nondividing state termed replicative senescence, which is characterized by altered gene expression (Hayflick et al. (1961) Exp. Cell Res. 25:585; Wright et al. (1989) Mol. Cell. Biol. 9:3088; Goldstein, (1990) Science 249:112; Campisi, (1996) Cell 84:497; Campisi (1997) Eur. J. Cancer 33:703; Faragher et al. (1997) Drug Discovery Today 2:64). Historically, replicative senescence has been viewed as being dependent upon cumulative cell divisions and not chronologic or metabolic time, indicating that proliferation is limited by a "mitotic clock" (Dell'Orco et al. (1973) Exp. Cell Res. 77:356; Hadey et al. (1978) J. Cell. Physiol. 97:509). The reduction in proliferative capacity of cells from old donors and patients with premature aging syndromes (Martin et al. (1970) Lab. Invest 23:86; Schneider et al. (1976) PNAS 73:3584; Schneider et al. (1972) Proc. Soc. Exp. Biol. Med. 141:1092; Elmore et al. (1976) Cell Physiol. 87:229), and the accumulation in vivo of senescent cells with altered patterns of gene expression (Stanulis-Praeger et al. (1987) Mech. Ageing Dev. 38:1; and Dimri et al. (1995) PNAS 92:9363), implicate cellular senescence in aging and age-related pathologies ((Hayflick et al. (1961) Exp. Cell Res. 25:585; Wright et al. (1989) Mol. Cell. Biol. 9:3088; Goldstein, (1990) Science 249:112; Campisi, (1996) Cell 84:497; Campisi (1997) Eur. J. Cancer 33:703; Faragher et al. (1997) Drug Discovery Today 2:64).

Cellular senescence is believed to contribute to multiple conditions in the elderly that could in principle be remedied by cell life-span extension in situ. Examples include atrophy of the skin through loss of extracellular matrix homeostasis in dermal fibroblasts; age-related macular degeneration caused by accumulation of lipofuscin and downregulation of a neuronal survival factor in RPE cells; and atherosclerosis caused by loss of proliferative capacity and overexpression of hypertensive and thrombotic factors in endothelial cells.

Extended life-span cells also have potential applications ex vivo. Cloned normal diploid cells could replace established tumor cell lines in studies of biochemical and physiological aspects of growth and differentiation; long-lived normal human cells could be used for the production of normal or engineered biotechnology products; and expanded populations of normal or genetically engineered rejuvenated cells could be used for autologous or allogeneic cell and gene therapy. Thus the ability to extend cellular life-span, while maintaining the diploid status, growth characteristics, and gene expression pattern typical of young normal cells, has important implications for biological research, the pharmaceutical

industry, and medicine.

Telomere loss is thought to be one aspect of the control of entry into senescence. Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome ends; these repeats are synthesized by the ribonucleoprotein enzyme telomerase. Telomerase is active in germline cells and, in humans, telomeres in these cells are maintained at about 15 kilobase pairs (kbp). In contrast, telomerase is not expressed in most human somatic tissues, and telomere length is significantly shorter. The telomere hypothesis of cellular aging proposes that cells become senescent when progressive telomere shortening during each division produces a threshold telomere length.

The human telomerase reverse transcriptase subunit (hTRT) has been cloned. See Nakamura et al., (1997) Science 277:955; Meyerson et al., (1997) Cell 90:78; and Kilian et al., (1997) Hum. Mol. Genet. 6:2011. It has recently been demonstrated that telomerase activity can be reconstituted by transient expression of hTRT in normal human diploid cells, which express the template RNA component of telomerase (hTR) but do not express hTRT. See, for example, Wang et al. (1998) Genes Dev 12:1769; and Weinrich et al., (1997) Nature Genet. 17:498. This provided the opportunity to manipulate telomere length and test the hypothesis that telomere shortening causes cellular senescence.

The reported results indicate that telomere loss in the absence of telomerase is the intrinsic timing mechanism that controls the number of cell divisions prior to senescence. The long-term effects of exogenous telomerase expression on telomere maintenance and the life-span of these cells remain to be determined in studies of longer duration.

Telomere homeostasis is likely to result from a balance of lengthening and shortening activities. Very low levels of telomerase activity are apparently insufficient to prevent telomere shortening. This is consistent with the observation that stem cells have low but detectable telomerase activity, yet continue to exhibit shortening of their telomeres throughout life. Thus, a threshold level of telomerase activity is likely required for life-span extension.

While the repair of telomers, e.g., by the activation of telomerase activity, can be enough for extending the replicative capacity of a cell, it can be a transforming event (e.g., to cause crisis and emergence of cancer cells), particularly where activation persists.

Summary of the Invention

One aspect of the present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In general, the subject method relies on the activation of a telomerase activity and inhibition of one or both of an Rb/p16 pathway or a p53 pathway.

The subject method is useful both *in vivo*, *ex vivo* and *in situ*. Exemplary uses include, merely to illustrate, the extension of stem cell or progenitor cell cultures or implants, the extension of skin or other epithelial cell cultures or grafts, the expansion of mesenchymal cell cultures or grafts, and the expansion of chondrocyte or osteocyte cultures or grafts. Exemplary stem and progenitor cells which can be extended by the subject method include neuronal, hematopoietic, epithelial, pancreatic, hepatic, chondrocytic and osteocytic stem and progenitor cells.

The subject method can be used for wound healing and other tissue repair, as well as cosmetic uses. It can be applied for prolonging the lifespan of a culture of normal cells or tissue being used to secrete therapeutic or other commercially significant proteins and products.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Brief Description of the Drawings

Figure 1. HEST2 encodes a human homolog of Est2p and p123. Alignment of the predicted amino acid sequence of HEST2 with the yeast Est2p and Euplotes p123 homologs. Amino residues within shaded and closed blocks are identical between at least two proteins. Identical amino acids within the RT motifs are in closed boxes, an example of a telomerase-specific motif in an outlined shaded box, and all identical amino acids in shaded boxes. RT motifs are extended in some cases to include other adjacent invariant or conserved amino acids. The sequence of the expressed tag AA281296 is underlined.

Figure 2. Alignment of RT motifs 1-6 of telomerase subunits HEST2, p123 and Est2p with S Cerevisiae group II intron-encoded RTs a2-Sc and a1-Sc. The consensus sequence of each RT motif is shown (h=hydrophobic, p=small polar, c=charged). Amino acids that are invariant among the telomerases and the RT consensus are in shaded boxes. Open boxes identify highly conserved residues unique to either telomerases or to nontelomerase RTs. Asterisks denote amino acids essential for polymerase catalytic function.

Figure 3. *Myc* activation of telomerase in HMEC cells. Primary HMEC cells at passage 12 were infected with empty vector (lanes 1-5), E6 (lanes 6-10), c-*myc* (lanes 11-15) or cdc25A (lanes 16-20) viruses. Two breast cancer cell lines BT549 (lanes 21-25) and T47D (lanes 26-30) were included for comparison. The cells were lysed and TRAP assays were performed using extract corresponding to 10,000 cells (lanes 2, 6, 7, 11,12, 17, 21, 22, 26 and 27), 1,000 cells (lanes 3, 8, 13, 18, 23 and 28), 100 cells (lanes 4, 9, 14, 19, 24 and 29) or 10 cells (lanes 5, 10, 15, 20, 25 and 30). Telomerase activity was shown to be sensitive to RNase by the addition of RNase A prior to the telomerase assay ("-", without RNase A; "+", with RNase A). To rule out the presence of inhibitors in apparently negative lysates, lanes labeled "Mix" (lanes 1 and 16) are assays containing lysate from 10,000 of the indicated cells mixed with lysate from 10,000 positive (c-*myc*-expressing) cells .

Figure 4. *Myc* activator of telomerase in IMR90 fibroblasts. IMR90 cells at passage 14 were infected with empty vector (lanes 1-5), c-*myc* (lanes 6-10) and E6 (lanes 11-15) viruses. HT1080 cells (lanes 15-20) were included for comparison. TRAP assays contained 10,000 cells (lanes 2, 6, 7, 12, 16 and 17), 1,000 cells (lanes 3, 8, 13 and 18), 100 cells (lanes 4, 9, 14 and 19) or 10 cells (lanes 5, 10, 15 and 20). Telomerase activity was shown to be sensitive to RNase by the addition of RNase A prior to extension reaction ("-", without RNase A; "+", with RNase A). "Mix" lanes (1 and 11) are assays containing lysate from 10,000 of the indicated cells mixed with lysate from 10,000 positive (c-*myc*-expressing) cells.

Figure 5. E6 increases c-*myc* protein level in HMEC. **A.** Levels of *myc* protein were determined by western blotting with a polyclonal *myc* antibody. Cell lysates from E6 (lane 1) and vector (lane 2) infected IMR90 cells and lysates from c-*myc* (lane 3), E6 (lane 4) and vector (lane 5) infected HMEC cells were analyzed. Tumor cell lines, HT1080 (lane 6),

HBL100 (Lane 7), BT549 (lane 8) and T47D (lane 9), were included for comparison. The expression of TFIIB was used to normalize loading. **B.** Total RNA prepared in parallel with the protein extracts used in **A.** was used in northern blots to determine *myc* mRNA levels. Equal quantities of total RNA, as indicated, were probed with a human c-*myc* cDNA.

Figure 6. Extension of telomere length and cellular lifespan by telomerase activation. **A.** Total RNA was prepared from normal HMEC and from HMEC that had been infected with a *myc* retrovirus. hEST2 transcript was visualized in equal quantities of RNA (10 μg) using a probe derived from the hEST2 cDNA. **B.** HMEC and IMR90 cells were infected with either empty vector (lanes 1-5 and 11-15) or hEST2 (lanes 6-10 and 16-20) viruses. TRAP assays were performed using lysate equivalent to 10,000 cells (lanes 2, 6, 7, 12, 16 and17), 1,000 cells (lanes 3, 8, 13 and 18), 100 cells (lanes 4, 9, 14 and 19) or 10 cells (lanes 5, 10, 15 and 20). Telomerase activity was shown to be sensitive to RNase by the addition of RNase A prior to assay ("-", without RNase A; "+", with RNase A). To rule out the presence of inhibitors in apparently negative lysates, lanes labeled "Mix" (lanes 1 and 16) are assays containing lysate from 10,000 of the indicated cells mixed with lysate from 10,000 positive (HT1080) cells. **C.** Genomic DNA from early passage HMEC (passage 12, lane 1), late passage HMEC (passage 22, lane 2), HMEC/hEST2 (cells infected at passage 12 with hEST2 and subsequently cultured for 10 additional passages, lane 3) and HMEC/vector (cells infected at passage 12 with empty vector and subsequently cultured for 10 additional passages, lane 4) were digested with *Rsa* I and *Hinf* I. Fragments were separated on a 0.8% agarose gel, and telomeric restriction fragments were visualized using a ^{32}P-labeled human telomeric sequence $(TTAGGG)_3$ as a probe. **D.** HMEC cells were transduced at passage 12 with either empty vector, c-*Myc* or hEST2 retroviruses (as indicated). These cells were continuously subcultured at a density of $4\text{-}5x10^5$ cells per 100 cm^2 once per week. After 12 passages following transduction, vector-infected cells could no longer be subcultured at this frequency and adopted a classic senescent phenotype. In contrast, cells expressing *myc* and hEST2 continue to proliferate and showed a virtual absence of senescent cells in the population.

Figure 7. Illustrates a MarxII vector including the coding sequence for hEST2. The long terminal repeats (LTRs) include, though not shown, recombinase sites such that, upon treatment of a cell in which the MarxII-hEST2 vector is integrated, the proviral vector including the hEST2 coding sequence is excised.

Detailed Description of the Invention

(i) Overview

The use of ex vivo cell-based therapies for treatment of various diseases is becoming increasingly prevalent. For example, expansion of a patient's own cells for skin grafting and cartilage repair is now commercially available. As the ability to manipulate primary cells evolves, other therapeutic intervention such as infusion of β-islet cells for the treatment of early- or late-onset diabetes or transplantation of neuronal precursor cells for neurodegenerative diseases such as Parkinson's disease are entering clinical trials. Genetic modification of a patient's cells ex vivo is also envisaged in the art as ultimately being useful for routine correction of inborn enzymatic deficiencies, establishment of disease resistance, or in the case of autoimmune disease, establishment of immune tolerance.

Cell-based therapies depend on the ability to isolate primary cell populations and to efficiently expand these cells in culture. However, normal human cells can execute only a limited number of divisions before entering an irreversible state of growth arrest, termed "replicative senescence". Hayflick, supra. For many years, techniques for establishing clonal populations of human cells have either begun with tumor cells or have required the introduction of genetic alterations that recaptiulate aspects of neoplastic transformation. Specific techniques for extending the lifespan of human cells in culture have involved introduction of viral antigens such SV40 T antigen, human papillomavirus antigens E6 and E7, or the adenovirus E1A or E1B antigens.

As set out above, this state can be triggered by a mortality control that is linked to telomere length. Indeed, reactivation of the telomerase enzyme can increase the proliferative potential of some cell populations. See, for example, Bodnar et al. (1998) Science 279:349; and Wang et al., supra.

However, as described in further detail below, indefinite expansion of normal cells in culture requires bypass of multiple mortality controls -- not just activation of telomerase. It was initially reported that this intervention extended lifespan without promoting transformation. In contrast, our data and that of others demonstrates that telomerase can only immortalize cells in conjunction with inactivation of the Rb/INK4 pathway. Immortalization by telomere extension requires that cells have already escaped a primary control point, M0. Spontaneous escape from M0 is accompanied by inactivation of the Rb/p16 tumor suppressor pathway. Indeed, applicants have observed that inactivation of the Rb/Ink4 pathway, particularly the Rb/p16INK4a pathway, occurs by spontaneous mutation in a significantly large percentage of tumors. Moreover, activation of telomerase alone selects for this event, e.g., Rb/Ink4 inactivation. Thus, because telomerase activation alone is a strongly pro-oncogenic selection, it would be imprudent to prepare cells for *ex vivo* therapy by a protocol based solely on extended activation of telomerase activity.

In one aspect, the present invention provides a method for increasing the proliferative

capacity of cells, preferably normal mammalian cells, by reversible immortalization in a way that preserves the genetic integrity of the normal cell. This is accomplished by a method which includes reversible inactivation M0 signals, and, in some instances, reversible activation of telomerase activity and/or reversible inactivation of apoptosis pathways. Following expansion of the modified normal cells, M0 control, and as appropriate, telomerase activity and apoptosis control, are returned to their normal states in order to yield a normal, mortal cell population. In preferred embodiments, the cells are isolated in culture for at least a portion of the treatment.

In general, one embodiment of the invention provides a method for increasing the proliferative capacity of metazoan cells, preferably mammalian cells, and more preferably normal mammalian cells, by contacting the cell with an agent that inactivates the antiproliferative activity of the Rb/INK4 pathway in the cell. Such agents are collectively referred to herein as "Rb inactivators".

In certain embodiments, the subject method relies on the ectopic expression of an "Rb dominant negative" form of a protein which is involved in the Rb pathway. Such proteins will include dominant negative forms of Rb, p16INK4a or other protein in the pathway whose wild-type allele inhibits proliferation when the Rb pathway is active.

In other embodiments, rather than using dominant negative mutants, the method relies on ectopic expression of a protein product which can selectively and reversibly inactivate an Rb/INK4 pathway, preferably an Rb/p16INK4a pathway, by a mechanism of agonizing Rb-dependent proliferation other than by negatively interfering with its own wild-type allele. Such proteins, e.g., which can bypass Rb, include MDM2 and the papillomavirus E7 protein. For use herein, the term "Rb inactivator" will also refer to mutations of a protein which ordinarily provides a proliferative signal that is negatively regulated by Rb, e.g., to remove Rb-dependent regulation of the proliferative activity of the protein.

In one sense, the Rb inactivator refers to any form of a protein, e.g., derived by point mutation, truncation, constitutive activation, etc, which prevents Rb-dependent inhibition of cyclin-dependent kinase (cdk) activity, especially G_1 cdks such as cdk4 or cdk6.

In yet other embodiments, the Rb inactivator is an antisense molecule or nucleic acid decoy.

In other embodiments, the Rb inactivator is a small molecule inhibitors of Rb or p16 function.

In preferred embodiments, the method is carried out such that inactivation of Rb is transient, and can be readily reversed. As described in greater detail below, such reversibility can be accomplished, as appropriately, by use of an excisable vector, an inducible

transcriptional regulatory element, an inducible Rb inactivator protein, application of the Rb inactivator in a paracrine form, or the use of small molecule agents, to name but a few.

Another aspect of the invention pertains to the Applicants' discovery that replicative senescence can also include a causative component regulated by a *ras*-dependent pathway. Thus, the present invention provides a method for increasing the proliferative capacity of normal cells which includes reversible inactivation of ras signaling in a way that preserves the genetic integrity of the genome of the host cell. As illustrate below, the ras inhibitor can be an agent which inhibits a ras/Raf/MKK/MAP kinase pathway, particularly one which can be added to cell culture. In certain embodiments, the agent inhibits ras activation, e.g., by inhibiting prenylation of ras or inhibiting the GTPases activity of ras. In other embodiments, the subject method utilizes an inhibitor of the kinase activity of raf, an MKK (Map kinase kinase) or a MAP kinase.

In certain embodiments, the Rb inactivator and the ras inhibitor are the same agent. In other embodiments, the Rb inactivator and the ras inhibitor are different agents.

In another aspect, the method of the present invention does not pre-suppose that there is a single genetic alteration that will extend the lifespan of most human cells in culture without selection for additional genetic mutations. Rather, in certain embodiments of the subject method, the cells are treated with an Rb inactivator and/or a ras inhibitor, along with an agent which selectively and reversibly activates a telomerase activity in the cell.

One aspect of the present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In preferred embodiments, the cells are isolated in culture for at least a portion of the treatment.

The subject methods are useful both *in vivo*, *ex vivo* and *in situ*. Exemplary uses include, merely to illustrate, the extension of stem cell or progenitor cell cultures or implants, the extension of skin or other epithelial cell cultures or grafts, the expansion of mesenchymal cell cultures or grafts, and the expansion of chondrocyte or osteocyte cultures or grafts. Exemplary stem and progenitor cells which can be extended by the subject method include neuronal, hematopoietic, pancreatic, and hepatic stem and progenitor cells.

An important feature of certain preferred embodiments of the subject method is the reversibility of inactivation or Rb and/or ras, and (optionally) activation of telomerase activity, rather than constitutive inactivation or activation (as the case may be). For example, where a vector is used to ectopically express an Rb inactivator or telomerase activator, the vector can be configured so as to be excisable from the cell. Thus, for *ex vivo* therapies, cells can be treated *ex vivo* with a vector encoding the appropriate protein or antisense, and prior to implantation, the vector can be excised to inhibit further recombinant expression of the

construct *in vivo*. In preferred embodiments, the vector can be excised so as to have little to no heterologous nucleic acid sequences in the host cell.

Thus, in one embodiment, cells are isolated from a donor animal, preferably a human, and treated with an Rb inactivator in an amount sufficient for at least 50 percent of the cells in the sample to avert replicative senescence, e.g., to proliferate beyond M0. The Rb inactivator is preferably chosen such that its activity, e.g., as an inhibitor of Rb-dependent senescence, is reversible, and more preferably is reversible because either (i) it is a gene construct, the expression of which, or presence in the cell, is inducible and/or reversible, (ii) its activity as an Rb Inhibitor is inducible and/or conditional, and/or (iii) it has a half-life in the cultured cells which does not extend far beyond the reimplantation of the cells, e.g., it preferably has a half-life of 2-20 population doublings. In certain embodiments, the cells are also treated with a ras inhibitor, preferably a ras inhibitor which is reversible. The ras inhibitor may also serve as an Rb inactivator. In certain embodiments, the cells are also treated with a telomerase activator, and preferably with a telomerase activator which is reversible. In certain embodiments, the cells are also treated with an apoptosis inhibitor, preferably an apoptosis inhibitor which is reversible. The addition of ras inhibitors, telomerase activators and apoptosis inhibitors can be individually in addition to the Rb inactivator, or various combination thereof.

The cells, after undergoing expansion in culture, are introduced into a host animal, which may the same or different than the donor animal. Prior to transplantation, or shortly thereafter, the Rb inactivator, ras inhibitor, telomerase activator and apoptosis inhibitor, as appropriate, are inactivated or otherwise remove from the cells.

Another aspect of the present invention relates to *in vitro* preparations of cells which have been treated by the subject method. Such cell compositions can be used, e.g., to generate a medicament for transplantation to an animal.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and

M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

(ii) Definitions

For convenience, certain terms used herein as defined below.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide, including both exonic and (optionally) intronic sequences. A gene, according to the present invention, can be in the form of a DNA construct which is transcribed or an RNA construct which is directly translatable.

By "ectopic expression", it is meant that a cell is caused to express, e.g., by expression of a heterologous or endogenous gene or by transcellular uptake of a protein, a higher than normal level of a particular protein (or RNA as the case may be) than the cell normally would for the particular starting phenotype.

As used herein, the term "transfection" means the introduction of a heterologous nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein with respect to transfected nucleic acid, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a polypeptide.

"Expression vector" refers to a replicable DNA construct used to express DNA which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a DNA sequence encoding a desired protein which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which

refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

In the expression vectors, regulatory elements controlling transcription or translation can be generally derived from mammalian, microbial, viral or insect genes The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. Vectors derived from viruses, such as retroviruses, adenoviruses, and the like, may be employed.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters and the like which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of the gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of one of the naturally-occurring forms of a protein.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of a urogenital origin, e.g. renal cells, or cells of a neural origin, e.g. neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a promoter or other transcriptional regulatory sequence is operably linked to a coding sequence if it controls the transcription of the coding sequence.

As used herein, the term "fusion protein" is art recognized and refer to a chimeric protein which is at least initially expressed as single chain protein comprised of amino acid sequences derived from two or more different proteins, e.g., the fusion protein is a gene product of a fusion gene.

The art term "fusion gene" refers to a nucleic acid in which two or more genes are fused resulting in a single open reading frame for coding two or more proteins that as a result of this fusion are joined by one or more peptide bonds.

The term "percent identical" refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

As used herein, the term "stringent conditions", when referring to hybridization conditions e.g., equivalent to about 20-27°C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt). In preferred embodiments, high stringency conditions are 0.2 x SSC at 50°C to 0.1 x SSC at 65°C.

The term "small organic molecule" refers to a non-peptide, non-nucleotide organic compound having a molecular weight less than 7500amu, more preferably less than 2500amu, and even more preferably less than 750amu.

The terms "EST2 proteins" and "EST2 polypeptides" refer to catalytic subunits of telomerase, preferably of a mammalian telomerase, and even more preferably of a human telomerase. Exemplary EST2 proteins are encoded by the nucleic acid of SEQ ID No:1, or by a nucleic acid which hybridizes thereto. Thus, the EST2 proteins useful in the subject method can be at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or even at least 95% identical to the human EST2 of SEQ ID No:2, or a fragment thereof which reconstitutes a telomerase elongation enzyme in a host cell (such as a human cell). A variety of different techniques are available in the art for assessing the activity of a particular EST2 polypeptide, e.g., which may vary in sequence and/or length relative to SEQ ID No: 1.

The term "telomerase-activating therapeutic agent" refers to any agent which can be used to activation of telomerase activity in a cell, e.g., a mammalian cell. For example, it includes expression vectors encoding EST2, *myc*, E6 or the like, formulations of such polypeptides, small molecule activators of expression of an endogenous telomerase activator gene, inhibitors of degradation of a telomerase activator, to name but a few.

The term "EST2 therapeutic agent" refers to any telomerase-activating therapeutic agent which can be used to cause ectopic expression of an EST2 polypeptide in a cell. For example, it includes EST2 expression vectors, formulations of EST2 polypeptides, and small molecule activators of expression of an endogenous EST2 gene, to name but a few.

The term "derepresses *myc*" refers to the ability of an agent to overcome an antagonism of *myc*, e.g., it may prevent mad/max inactivation of *myc* and thereby activates *myc*.

The term "progenitor cell" refers to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. As used herein, the term "progenitor cell" is also intended to encompass a cell which is sometimes referred to in the art as a "stem cell". In a preferred embodiment, the term "progenitor cell" refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues.

As used herein, the term "cellular composition" refers to a preparation of cells, which preparation may include, in addition to the cells, non-cellular components such as cell culture media, e.g. proteins, amino acids, nucleic acids, nucleotides, co-enzyme, anti-oxidants, metals and the like. Furthermore, the cellular composition can have components which do not affect the growth or viability of the cellular component, but which are used to provide the cells in a particular format, e.g., as polymeric matrix for encapsulation or a pharmaceutical preparation.

As used herein the term "substantially pure", with respect to progenitor cells, refers to a population of progenitor cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to progenitor cells making up a total cell population. Recast, the term "substantially pure" refers to a population of progenitor cell of the present invention that contain fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5%, of lineage committed cells in the original unamplified and isolated population prior to subsequent culturing and amplification.

The term "cosmetic preparation" refers to a form of a pharmaceutical preparation which is formulated for topical administration.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

(iii) Inactivation of M0 Control

Upon explant into culture, primary human epithelial cells and keratinocytes have a replicative lifespan of only a few population doublings, following which cells enter a growth-arrested state termed M0 (Foster et al. (1996) Oncogene 12:1773). In many ways, M0 cells resemble cells that senesce in M1; however, M0 is not triggered by telomere depletion. Activation of telomerase in pre-M0 human mammary epithelial cells (HMEC) or keratinocytes does not prevent M0 arrest.

M0 has also been called "selection". Although the bulk of a primary cell population arrests at M0, in certain cell types (e.g., HMEC) a few cells escape to yield a "normal" cell strain that is capable of further proliferation. Thus, the M0 event was long considered to be the ill-defined process of "culture adaptation". However, this perception has changed with the realization that escape from M0 selects for specific oncogenic mutations.

A. Inactivation of the Rb/INK4 pathway(s)

The retinoblastoma gene product, Rb, is a component of normal cell growth. In the hypophosphorylated state, Rb prevents the activation of genes needed for cell cycle progression. For review, see for example, Sherr et al. (1996) Science 274:1672. During the G1 phase of the cell-cycle, phosphorylation of Rb by two closely related cyclin-dependent kinases, cdk4 and cdk6, releases the cells into the division cycle. These cdk's are regulated by association with stimulatory and inhibitory subunits. Binding of INK4 family members, such as the tumor suppressor p16INK4a, inhibits kinase activity and thereby prevents Rb phosphorylation, with the result being growth arrest. According to the present invention, the proliferative capacity of cells, especially cultured human cells, can be increased by contacting the cells with an "Rb inactivator" which can selectively and reversibly inactivate an Rb/INK4 pathway, preferably an Rb/p16INK4a pathway

In one embodiment, the subject method comprises delivering into the cell an expression construct encoding an "Rb inactivator", e.g., a polypeptide or nucleic acid which

can inactivate an Rb/INK4 pathway, preferably an Rb/p16INK4a pathway.

In certain embodiments, such constructs encode a polypeptide. For example, the expression construct can include coding sequences for MDM2. The MDM2 oncoprotein is a cellular inhibitor of both Rb/E2F function and the p53 tumor suppressor. In certain cancers, MDM2 amplification is a common event and contributes to the inactivation of Rb and/or p53. Preferred human MDM2 sequences are provided at SWISS-PROT locus MDM2_HUMAN, accession Q00987 and GenBank accession U33201. The MDM2 coding sequence can encode an MDM2 protein, or fragment thereof which retains an inhibitory activity over p53 and/or Rb growth suppression, which is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with a sequence of SEQ ID No. 4, or which is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 3. For example, such fragments of MDM2 which may be useful in the present method include the p53-binding domain, as well as an inhibitory domain that can directly repress basal transcription in the absence of p53, e.g., which includes amino acids 50-222 of MDM2. See, for example, Thut et al. (1997) Genes Dev 11:1974.

In other embodiments, the construct encodes a dominant negative cdk4 or cdk6 mutants, e.g., which has lost the ability to bind and/or be inhibited by an INK4 protein, especially p16INK4a. Exemplary CDK mutants of this type are described in USSN 08/581,918. Such forms of cdk4 and cdk6 are based on the observations that mutants of cdk4 have been identified in cells from a melanoma patient and which were constitutively activate in that their activity was independent of p16INK4a.

As demonstrated in USSN 08/581,918, this and other mutations to the cdk4 and cdk6 sequences can cause constitutive activation by abrogating p16INK4a binding to the mutant cdk. For instance, when these changes were visualized onto the 3-dimensional structure, it was apparent that these residues form a cluster of four amino acid residues accessible to solvent. These residues, K22, R24, H95 and D97 define a surface in the small lobe of cdk4, in very close proximity to the ATP binding site, but far away from the cyclin binding site or the substrate binding site. This surface likely represents at least a portion of the p16/p15-recognition surface present in cdk4 (and homologously in cdk6). Accordingly, an attractive model for p16/p15 inhibition of cdk4/cdk6 provides an occlusion or distorting effect to the ATP-binding site upon binding of the CCR protein such that ATP either does not bind to cdk4 or is not properly positioned to be used as a phosphate donor.

Thus, in certain embodiments, the subject method provides an expression construct encoding a cdk4 mutant that is different by one or more amino acid residues from SEQ ID No. 6, and that has a p16INK4a-independent kinase activity, e.g., having a coding sequence which hybridizes to the coding sequence of SEQ ID No. 5, e.g., having an amino acid sequence that

is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with a sequence of SEQ ID No. 6. In preferred embodiments, the subject cdk4 protein has an amino acid sequence which differs at one or more of K22, R24, H95 and/or D97 from SEQ ID No. 6.

In still other embodiments, the construct encodes a dominant negative Rb mutant. For instance, wild-type retinoblastoma protein function can be disrupted by expression of its C pocket fragment. See, for example Welch et al. (1995) Genes Dev 9:31-46. Other dominant negative mutants are described in, e.g., Muthukkumar et al. (1996) J Biol Chem 271: 5733-40.

In still other embodiments, the construct can encode a papillomavirus E7 protein, or other viral oncoprotein which bypasses Rb and/or p53, or fragment thereof. In preferred embodiments, the E7 protein is from a high risk HPV, preferably HPV-16 or HPV-18. An exemplary E7 polypeptide is provided by SWISS-PROT: locus VE7_HPV18, accession P06788, PIR locus W7WL18 and SEQ ID No. 7.

In still other embodiments, the construct encodes a cyclin, preferably a cyclin active in G1 phase, such as cyclin D1 or cyclin E.

In yet other embodiments, the RB inactivator can be a transcriptional repressor, or dominant negative mutant of a transcriptional activator, which inhibits expression of Rb, an INK4 protein (such as p16INK4a) or other positive regulator of Rb antiproliferative activity. In one embodiment, the Rb inactivator is the Bmi-1 gene product. Jacobs et al. (1999) Nature identifies Bmi-1 as a transcriptional repressor of the polycomb-group which inhibits expression of $p16^{INK4a}$ and $p19^{Arf}$. An exemplary human Bim-1 is given in GenBank as accession L13689.

Moreover, the art is replete with examples of combinatorial techniques for identifying mutants, e.g., point mutations, additions, deletions and fragments of any of the above-referenced polypeptides which retain a particular biological activity with respect to bypassing the Rb checkpoint. Combinatorial techniques for generating and processing libraries of variants of a protein are routine in the art, even for libraries exceeding a billion different variants. Exemplary mutagenic techniques include alanine scanning mutagenesis and the like (Lowman et al. (1991) *Biochemistry* 30:10832-10838; and Cunningham et al. (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Brown et al. (1992) *Mol. Cell Biol.* 12:2644-2652; McKnight et al. (1982) *Science* 232:316); by saturation mutagenesis (Meyers et al. (1986) *Science* 232:613); by PCR mutagenesis (Leung et al. (1989) *Method Cell Mol Biol* 1:11-19); and by random mutagenesis (Miller et al. (1992) *A Short Course in Bacterial Genetics*, CSHL Press, Cold Spring Harbor, NY), which can be used to create libraries of variants which can be screened for a given biological activity.

Gallop et al. (1994) *J Med Chem* 37:1233 further illustrates the state of the art. In

particular, Gallop et al. describe the general state of the art of combinatorial libraries for screening analog libraries to determine the minimum size of the active sequence and for identifying those residues critical for binding and intolerant of substitution. In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of variants which can be rapidly screened to identify variants/fragments which retained Rb inactivator activity.

It is plain from the combinatorial mutagenesis art that it would in fact be routine for those skilled in the art to engage in large scale mutagenesis of proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughout analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

In yet another embodiment, the construct "encodes" an antisense molecule which inhibits p16 or Rb expression. For instance, the antisense construct includes a nucleotide sequence that hybridizes under stringent conditions to a p16INK4a gene or an Rb gene, preferably a mammalian gene, and even more preferably a human gene. In preferred embodiments, the antisense construct includes a nucleotide sequence that hybridizes to the Rb gene of SEQ ID No. 8 or GenBank Accession L41870, or to the p16INK4a gene of SEQ ID No. 9 or GenBank Accession L27211. In preferred embodiments, the antisense hybridizes to a coding sequence of an Rb or p16INK4a gene.

Other Rb inactivators are contemplated by the present invention. For instance, PCT publication WO 98/12339 and US patent application 09/031,185 describe techniques for detecting genes which interfere with such Rb/p16 phenotypes. For example, genes which can bypass Rb/p16-mediated senescence can be examined by overexpression of sense orientation genes or by functional knock-out (expression of genetic suppressor elements). To illustrate, mouse embryo fibroblasts (MEF) which lack endogenous Rb genes (from Rb knock-out mice) are engineered to conditionally express a fluorescently tagged Rb protein. When activated, the fluorescent protein enforces cell cycle arrest. Bypass of the arrest can be accomplished by expression of sense cDNAs or by expression of GSE fragments. Such a screen might identify components of the Rb-degradative pathway, genes that do not affect Rb but allow cell cycle progression even in the presence of Rb and genes that affect Rb localization. Therefore, use of a fluorescent Rb protein provides information as to the mechanism of bypass.

Genes, or genetic suppressor elements, which are identified as permitting bypass of the Rb/p16 pathway can be delivered as part of the present method.

In accordance with the subject method, expression constructs of the subject Rb inactivator polypeptides or Rb inactivator nucleic acids (antisense, decoys, etc) may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *in vitro* or *in vivo* with a recombinant gene. Approaches include insertion of the subject Rb inactivator gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out in vivo. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for introduction of nucleic acid encoding an Rb inactivator into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes in vivo, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding, e.g., an Rb inactivator polypeptide, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory

manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

In choosing retroviral vectors as a gene delivery system for the subject Rb inactivator proteins, it is important to note that a prerequisite for the successful infection of target cells by most retroviruses, and therefore of stable introduction of the recombinant gene, is that the target cells must be dividing. In general, this requirement will not be a hindrance to use of retroviral vectors to deliver the subject gene constructs.

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al. (1989) PNAS 86:9079-9083; Julan et al. (1992) J. Gen Virol 73:3251-3255; and Goud et al. (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et al. (1991) J Biol Chem 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the recombinant gene of the retroviral vector.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interRb inactivator, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), and smooth muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) Cell 16:683; Berkner et al., supra; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. Pp. 109-127). Expression of the inserted gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject Rb inactivator constructs is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. Curr. Topics in Micro. And Immunol. (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 can be

used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistent expression of the subject Rb inactivator proteins in cells of the central nervous system, such as neuronal stem cells, and ocular tissue (Pepose et al. (1994) Invest Ophthalmol Vis Sci 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a the subject proteins in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding one of the subject proteins can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) No Shinkei Geka 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with monoclonal antibodies against glioma-associated antigen (Mizuno et al. (1992) Neurol. Med. Chir. 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, the subject gene construct can be used to transfect hepatocytic cells in vivo using a soluble polynucleotide carrier comprising an asialoglycoprotein conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of

DNA-containing endosomes (Mulligan et al. (1993) Science 260-926; Wagner et al. (1992) PNAS 89:7934; and Christiano et al. (1993) PNAS 90:2122).

In preferred embodiments of the present invention, the subject method comprises delivering into the cell a gene construct which can *selectively* and *reversibly* inactivate Rb activity in the cell.

In one embodiment, the coding sequence for the Rb inactivator is provided as part of a vector which can be partially or completely excised from the host cell is an inducible manner. For instance, the vector can include:

(i) one or more transposition elements for integration of the vector into chromosomal DNA of a eukaryotic host cell;

(ii) a coding sequence of an Rb inactivator; and

(iii) excision elements for removing, upon contact of the cell with an excision agent (which activates the excision element) all or at least the portion of an integrated form of the vector from chromosomal DNA in a manner which is results in loss-of-function of the heterologous Rb inactivator.

For example, the excision elements can be provided in the vector so as flank at least the coding sequence of an Rb inactivator, though they may flank only a portion of the coding sequence such that the sequence resulting after excision does not encode a functional activator, or they may flank a sufficient portion of a transcriptional regulatory sequence for the Rb inactivator such that resulting construct does not express the Rb inactivator.

In preferred embodiments, the excision elements are disposed in the vector such that, upon excision of the integrated form of the vector, no or substantially no portion (e.g., less than 200, 100 or 50 nucleotides) of the vector DNA is left in the chromosomal DNA of the host cell. In preferred embodiments, the vector DNA which remains in the genome is transcriptionally inert, e.g., it does not induce or repress transcription of proximally located genomic sequences.

In preferred embodiments, the transposition elements are viral transposition elements, e.g., retroviral or lentiviral transposition elements, such as may be provided where the vector is a replication-deficient virus.

In preferred embodiments, the excision elements comprise enzyme-assisted site-specific integration sequences. For instance, the excision elements may include recombinase target sites, e.g., recombinase target sites for Cre recombinase, Flp recombinase, Pin recombinase, lamda integrase, Gin recombinase, Kw recombinase, or R recombinase. The excision elements may also be restriction enzyme sites.

In preferred embodiments, the vector is a retroviral vector which recombinase sites which are located in the LTRs such that excision of a proviral sequence occurs, e.g., the viral vector is completely, or nearly completely excised from the chromosomal DNA of the host cell.

The vector can include such other elements as: transcriptional regulatory sequences for directing transcription of the coding sequence for the telemorase activator cnucleic; a packaging signal for packaging the vector in an infectious viral particle;

Exemplary vectors of this type, e.g., readily excisable, are described in the appended examples as well as PCT publication WO 98/12339. On advantage that certain of these vectors have, e.g., those which can be substantially excised, can be realized for embodiments wherein the method is part of an *ex vivo* therapy. In such embodiments, the cells can be treated *ex vivo* with the constructs. Prior to implantation in a host, the cells are treated with an agent, such as a recombinase, which results in excision of the vector from the genomic DNA of the host cell. Thus, the cells which are implanted are no longer genetically engineered. In such embodiments, it may be desirable to include one or more detectable genes (markers) on the vector in order to be able to identify cells which still retained the vector, e.g., by FACS sorting, affinity purification or other techniques.

The reversibility of Rb inactivation can also be generated by use of an expression system which is inducible because of the presence of an inducible transcriptional regulatory sequence controlling the expression of the coding sequence of the Rb inactivator. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. Where the cells are to be transplanted into a patient, the inducible promoter is preferably one which is regulated by a small molecule or other factor which is not endogenous to the host animal.

Exemplary regulatable promoters include the tetracycline responsive promoters, such as described in, for example, Gossen et al. (1992) PNAS 89:5547-5551; and Pescini et al., (1994) Biochem. Biophys. Res. Comm. 202:1664-1667.

In another embodiment, the subject method utilizes the multimerization technology first pioneered by Schreiber and Crabtree. This technique permits the regulation of expression of an endogenous or heterologous gene, in this case a coding sequence for Rb inactivator, by use of chimeric transcription factors which are dependent on small molecules "dimerizers" to assemble transcriptionally active complexes. See, for example, PCT publications WO 9612796; WO 9505389; WO 9502684; WO 9418317; WO 9606097; and WO 9606110. Moreover, a number of techniques have been developed more recently which permit the recruitment of endogenous DNA binding and activation domains to the transcriptional

regulatory sequences by use of artificial dimerization molecules. See, for example, PCT publication WO 9613613.

In other embodiments, the reversibility of Rb inactivation can be accomplished by use of conditionally active (or conditionally inactivable) forms of an Rb inactivator. For instance, temperature-sensitive mutants of a dominant negative inactivator, such as a cdk4 mutant, can be employed in the subject method. In embodiments wherein the cells are to be transplanted into an animal, the ts mutant can be inactive at body temperature (the non-permissive temperature) and active at a lower or higher cell culture temperature.

To illustrate, one strategy for producing a temperature-sensitive Rb inactivator, that does not require a search for a ts mutation in a gene of interest, is based on a portable, heat-inducible N-degron. The N-degron is an intracellular degradation signal whose essential determinant is a "destabilizing" N-terminal residue of a protein. A set of N-degrons containing different destabilizing residues is manifested as the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-degron consists of at least two determinants: a destabilizing N-terminal residue and a specific internal Lys residue (or residues) of a substrate. The Lys residue is the site of attachment of a multiubiquitin chain. Ubiquitin is a protein whose covalent conjugation to other proteins plays a role in a number of cellular processes, primarily through routes that involve protein degradation. For a description of exemplary heat-inducible N-degron modules which can be adapted for generating conditional mutants of an Rb inactivator, see US Patents 5,705,387 and 5,538,862, and Dohmen et al. (1994) Science 263:1273-6.

In yet another embodiment, ectopic expression of an Rb inactivator can be by way of a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous Rb inactivator gene. For instance, the gene activation construct can replace the endogenous promoter of an Rb inactivator gene, such as a bmi-1 gene, with a heterologous promoter, e.g., one which causes constitutive expression of the Rb inactivator gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

Such embodiments are useful wherein the Rb inactivator is a wild-type gene whose product, when overexpressed, bypasses the Rb checkpoint. For example, overexpression of cyclin D1, cdk4, cdk6, cdc25A, or bmi-1 can be used as a means for bypassing the antiproliferative activity of Rb.

The gene activation construct is inserted into a cell, and integrates with the genomic

DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with, e.g., the native Rb inactivator gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous Rb inactivator gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, J. Exp. Med., 169:13), the human β-actin promoter (Gunning et al. (1987) PNAS 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) Mol. Cell Biol. 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) Nature 290:304-310; Templeton et al. (1984) Mol. Cell Biol., 4:817; and Sprague et al. (1983) J. Virol., 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) PNAS 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) Nature Genetics, 1:379-384).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression.

In yet another embodiment, membrane permeable drugs (e.g., preferably small organic molecules) can be identified which activate the expression of an endogenous Rb inactivator gene.

In embodiments wherein the cells are treated in culture, RNA encoding Rb inactivator can be introduced directly into the cell, e.g., from RNA generated by *in vitro* transcription. In preferred embodiments, the RNA is preferably a modified polynucleotide which is resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases. Exemplary nucleic acid modifications which can be used to generate such RNA polynucleotides include phosphoramidate, phosphothioate and methylphosphonate analogs of nucleic acids (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775), or peptide nucleic acids (PNAs).

In still another embodiment of the subject method, the Rb inactivator polypeptide can be contacted with a cell under conditions wherein the protein is taken up by the cell, e.g., internalized, without the need for recombinant expression in the cell. For instance, in the application of the subject method to skin, mucosa and the like, a variety of techniques have been developed for the transcytotic delivery of ectopically added proteins.

In an exemplary embodiment, the Rb inactivator is provided for transmucosal or transdermal delivery. For such administration, penetrants appropriate to the barrier to be permeated are used in the formulation with the polypeptide. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the proteins of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. For example, Chien et al. (1989) J. Pharm. Sci. 78:376-383 describes direct current iontophoretic transdermal delivery of peptide and protein drugs. Srinivasan et al., (1989) J. Of Pharm. Sci. 78:370-375 describes the transdermal iontophoretic drug delivery : Mechanistic analysis and application to polypeptide delivery. See also USSN 4,940,456.

USSN 5,459,127 describes the use of cationic lipids for intracellular delivery of biologically active molecules.

USSN 5,190,762 describes methods of administering proteins to living skin cell.

In another embodiment, the polypeptide is provided as a chimeric polypeptide which includes a heterologous peptide sequence ("internalizing peptide") which drives the translocation of an extracellular form of a therapeutic polypeptide sequence across a cell membrane in order to facilitate intracellular localization of the therapeutic polypeptide. In this regard, the therapeutic polypeptide sequence is one which is active intracellularly, such as a tumor suppressor polypeptide, transcription factor or the like. The internalizing peptide, by itself, is capable of crossing a cellular membrane by, e.g., transcytosis, at a relatively high rate. The internalizing peptide is conjugated, e.g., as a fusion protein, to the Rb inactivator polypeptide. The resulting chimeric polypeptide is transported into cells at a higher rate

relative to the activator polypeptide alone to thereby provide an means for enhancing its introduction into cells to which it is applied, e.g., to enhance topical applications of the Rb inactivator polypeptide.

In one embodiment, the internalizing peptide is derived from the drosopholia antepennepedia protein, or homologs thereof. The 60 amino acid long long homeodomain of the homeo-protein antepennepedia has been demonstrated to translocate through biological membranes and can facilitate the translocation of heterologous polypeptides to which it is couples. See for example Derossi et al. (1994) J Biol Chem 269:10444-10450; and Perez et al. (1992) J Cell Sci 102:717-722. Recently, it has been demonstrated that fragments as small as 16 amino acids long of this protein are sufficient to drive internalization. See Derossi et al. (1996) J Biol Chem 271:18188-18193. The present invention contemplates a chimeric protein comprising at least one Rb inactivator polypeptide sequence and at least a portion of the antepennepedia protein (or homolog thereof) sufficient to increase the transmembrane transport of the chimeric protein, relative to the Rb inactivator polypeptide, by a statistically significant amount.

Another example of an internalizing peptide is the HIV transactivator (TAT) protein. This protein appears to be divided into four domains (Kuppuswamy et al. (1989) Nucl. Acids Res. 17:3551-3561). Purified TAT protein is taken up by cells in tissue culture (Frankel and Pabo, (1989) Cell 55:1189-1193), and peptides, such as the fragment corresponding to residues 37 -62 of TAT, are rapidly taken up by cell *in vitro* (Green and Loewenstein, (1989) Cell 55:1179-1188). The highly basic region mediates internalization and targeting of the internalizing moiety to the nucleus (Ruben et al., (1989) J. Virol. 63:1-8). Peptides or analogs that include a sequence present in the highly basic region, such as CFITKALGISYGRKKRRQRRRPPQGS, are conjugated to Rb inactivator polypeptides to aid in internalization and targeting those proteins to the intracellular milieu.

Another exemplary transcellular polypeptide can be generated to include a sufficient portion of mastoparan (T. Higashijima et al., (1990) J. Biol. Chem. 265:14176) to increase the transmembrane transport of the chimeric protein.

While not wishing to be bound by any particular theory, it is noted that hydrophilic polypeptides may be also be physiologically transported across the membrane barriers by coupling or conjugating the polypeptide to a transportable peptide which is capable of crossing the membrane by receptor-mediated transcytosis. Suitable internalizing peptides of this type can be generated using all or a portion of, e.g., a histone, insulin, transferrin, basic albumin, prolactin and insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II) or other growth factors. For instance, it has been found that an insulin fragment, showing affinity for the insulin receptor on capillary cells, and being less effective than insulin in blood sugar

reduction, is capable of transmembrane transport by receptor-mediated transcytosis and can therefor serve as an internalizing peptide for the subject transcellular polypeptides. Preferred growth factor-derived internalizing peptides include EGF (epidermal growth factor)-derived peptides, such as CMHIESLDSYTC and CMYIEALDKYAC; TGF- beta (transforming growth factor beta)-derived peptides; peptides derived from PDGF (platelet-derived growth factor) or PDGF-2; peptides derived from IGF-I (insulin-like growth factor) or IGF-II; and FGF (fibroblast growth factor)-derived peptides.

Another class of translocating/internalizing peptides exhibits pH-dependent membrane binding. For an internalizing peptide that assumes a helical conformation at an acidic pH, the internalizing peptide acquires the property of amphiphilicity, e.g., it has both hydrophobic and hydrophilic interfaces. More specifically, within a pH range of approximately 5.0-5.5, an internalizing peptide forms an alpha-helical, amphiphilic structure that facilitates insertion of the moiety into a target membrane. An alpha-helix-inducing acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes. Such internalizing peptides can be used to facilitate transport of Rb inactivator polypeptides, taken up by an endocytic mechanism, from endosomal compartments to the cytoplasm.

A preferred pH-dependent membrane-binding internalizing peptide includes a high percentage of helix-forming residues, such as glutamate, methionine, alanine and leucine. In addition, a preferred internalizing peptide sequence includes ionizable residues having pKa's within the range of pH 5-7, so that a sufficient uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane.

A particularly preferred pH-dependent membrane-binding internalizing peptide in this regard is aa1-aa2-aa3-EAALA(EALA)4-EALEALAA-amide, which represents a modification of the peptide sequence of Subbarao et al. (Biochemistry 26:2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue, such as cysteine or lysine, that facilitates chemical conjugation of the internalizing peptide to a targeting protein conjugate. Amino acid residues 2-3 may be selected to modulate the affinity of the internalizing peptide for different membranes. For instance, if both residues 2 and 3 are lys or arg, the internalizing peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the internalizing peptide will insert into neutral membranes.

Yet other preferred internalizing peptides include peptides of apo-lipoprotein A-1 and B; peptide toxins, such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid hormone, pancreatic polypeptide; and peptides corresponding to signal sequences of numerous secreted proteins. In addition, exemplary

internalizing peptides may be modified through attachment of substituents that enhance the alpha-helical character of the internalizing peptide at acidic pH.

Yet another class of internalizing peptides suitable for use within the present invention include hydrophobic domains that are "hidden" at physiological pH, but are exposed in the low pH environment of the target cell endosome. Upon pH-induced unfolding and exposure of the hydrophobic domain, the moiety binds to lipid bilayers and effects translocation of the covalently linked polypeptide into the cell cytoplasm. Such internalizing peptides may be modeled after sequences identified in, e.g., Pseudomonas exotoxin A, clathrin, or Diphtheria toxin.

Pore-forming proteins or peptides may also serve as internalizing peptides herein. Pore-forming proteins or peptides may be obtained or derived from, for example, C9 complement protein, cytolytic T-cell molecules or NK-cell molecules. These moieties are capable of forming ring-like structures in membranes, thereby allowing transport of attached polypeptide through the membrane and into the cell interior.

Mere membrane intercalation of an internalizing peptide may be sufficient for translocation of the polypeptide, e.g. an Rb inactivator protein, across cell membranes. However, translocation may be improved by attaching to the internalizing peptide a substrate for intracellular enzymes (i.e., an "accessory peptide"). It is preferred that an accessory peptide be attached to a portion(s) of the internalizing peptide that protrudes through the cell membrane to the cytoplasmic face. The accessory peptide may be advantageously attached to one terminus of a translocating/internalizing moiety or anchoring peptide. An accessory moiety of the present invention may contain one or more amino acid residues. In one embodiment, an accessory moiety may provide a substrate for cellular phosphorylation (for instance, the accessory peptide may contain a tyrosine residue).

An exemplary accessory moiety in this regard would be a peptide substrate for N-myristoyl transferase, such as GNAAAARR (Eubanks et al., in: Peptides. Chemistry and Biology, Garland Marshall (ed.), ESCOM, Leiden, 1988, pp. 566-69) In this construct, an internalizing, peptide would be attached to the C-terminus of the accessory peptide, since the N-terminal glycine is critical for the accessory moiety's activity. This hybrid peptide, upon attachment to an Rb inactivator polypeptide at its C-terminus, is N-myristylated and further anchored to the target cell membrane, e.g., it serves to increase the local concentration of the polypeptide at the cell membrane.

To further illustrate use of an accessory peptide, a phosphorylatable accessory peptide is first covalently attached to the C-terminus of an internalizing peptide and then incorporated into a fusion protein with an Rb inactivator polypeptide. The peptide component of the fusion protein intercalates into the target cell plasma membrane and, as a result, the accessory peptide

is translocated across the membrane and protrudes into the cytoplasm of the target cell. On the cytoplasmic side of the plasma membrane, the accessory peptide is phosphorylated by cellular kinases at neutral pH. Once phosphorylated, the accessory peptide acts to irreversibly anchor the fusion protein into the membrane. Localization to the cell surface membrane can enhance the translocation of the polypeptide into the cell cytoplasm.

Suitable accessory peptides include peptides that are kinase substrates, peptides that possess a single positive charge, and peptides that contain sequences which are glycosylated by membrane-bound glycotransferases. Accessory peptides that are glycosylated by membrane-bound glycotransferases may include the sequence x-NLT-x, where "x" may be another peptide, an amino acid, coupling agent or hydrophobic molecule, for example. When this hydrophobic tripeptide is incubated with microsomal vesicles, it crosses vesicular membranes, is glycosylated on the luminal side, and is entrapped within the vesicles due to its hydrophilicity (C. Hirschberg et al., (1987) Ann. Rev. Biochem. 56:63-87). Accessory peptides that contain the sequence x-NLT-x thus will enhance target cell retention of corresponding polypeptide.

In another embodiment of this aspect of the invention, an accessory peptide can be used to enhance interaction of the Rb inactivator polypeptide with the target cell. Exemplary accessory peptides in this regard include peptides derived from cell adhesion proteins containing the sequence "RGD", or peptides derived from laminin containing the sequence CDPGYIGSRC. Extracellular matrix glycoproteins, such as fibronectin and laminin, bind to cell surfaces through receptor-mediated processes. A tripeptide sequence, RGD, has been identified as necessary for binding to cell surface receptors. This sequence is present in fibronectin, vitronectin, C3bi of complement, von-Willebrand factor, EGF receptor, transforming growth factor beta , collagen type I, lambda receptor of E. Coli, fibrinogen and Sindbis coat protein (E. Ruoslahti, Ann. Rev. Biochem. 57:375-413, 1988). Cell surface receptors that recognize RGD sequences have been grouped into a superfamily of related proteins designated "integrins". Binding of "RGD peptides" to cell surface integrins will promote cell-surface retention, and ultimately translocation, of the polypeptide.

As described above, the internalizing and accessory peptides can each, independently, be added to an Rb inactivator polypeptide by either chemical cross-linking or in the form of a fusion protein. In the instance of fusion proteins, unstructured polypeptide linkers can be included between each of the peptide moieties.

In general, the internalization peptide will be sufficient to also direct export of the polypeptide. However, where an accessory peptide is provided, such as an RGD sequence, it may be necessary to include a secretion signal sequence to direct export of the fusion protein from its host cell. In preferred embodiments, the secretion signal sequence is located at the

extreme N-terminus, and is (optionally) flanked by a proteolytic site between the secretion signal and the rest of the fusion protein.

In an exemplary embodiment, an Rb inactivator polypeptide is engineered to include an integrin-binding RGD peptide/SV40 nuclear localization signal (see, for example Hart SL et al., 1994; J. Biol. Chem.,269:12468-12474), such as encoded by the nucleotide sequence provided in the Nde1-EcoR1 fragment: catatgggtggctgccgtggcgatatgttcggttgcggtgctcctccaaaaaagaagagaaag-gtagctggattc, which encodes the RGD/SV40 nucleotide sequence: MGGCRGDMFGCGAPP-KKKRKVAGF. In another embodiment, the protein can be engineered with the HIV-1 tat(1-72) polypeptide, e.g., as provided by the Nde1-EcoR1 fragment:catatggagccagtagatcctagactagagccc-tggaagcatccaggaagtcagcctaaaactgcttgtaccaattgctattgtaaaaagtgttgctttcattgccaagtttgtttcataacaaaag ccettggcatctcctatggcaggaagaagcggagacagcgacgaagacctcctcaaggcagtcagactcatcaagtttctctaagtaag caaggattc, which encodes the HIV-1 tat(1-72) peptide sequence: MEPVDPRLEPWKHPGSQPKT-ACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ. In still another embodiment, the fusion protein includes the HSV-1 VP22 polypeptide (Elliott G., O'Hare P (1997) Cell, 88:223-233) provided by the Nde1-EcoR1 fragment:

cat atg acc tct cgc cgc tcc gtg aag tcg ggt ccg cgg gag gtt ccg cgc gat gag tac gag gat ctg tac tac acc ccg tct tca ggt atg gcg agt ccc gat agt ccg cct gac acc tcc cgc cgt ggc gcc cta cag aca cgc tcg cgc cag agg ggc gag gtc cgt ttc gtc cag tac gac gag tcg gat tat gcc ctc tac ggg ggc tcg tca tcc gaa gac gac gaa cac ccg gag gtc ccc cgg acg cgg cgt ccc gtt tcc ggg gcg gtt ttg tcc ggc ccg ggg cct gcg cgg gcg cct ccg cca ccc gct ggg tcc gga ggg gcc gga cgc aca ccc acc acc gcc ccc cgg gcc ccc cga acc cag cgg gtg gcg act aag gcc ccc gcg gcc ccg gcg gcg gag acc acc cgc ggc agg aaa tcg gcc cag cca gaa tcc gcc gca ctc cca gac gcc ccc gcg tcg acg gcg cca acc cga tcc aag aca ccc gcg cag ggg ctg gcc aga aag ctg cac ttt agc acc gcc ccc cca aac ccc gac gcg cca tgg acc ccc cgg gtg gcc ggc ttt aac aag cgc gtc ttc tgc gcc gcg gtc ggg cgc ctg gcg gcc atg cat gcc cgg atg gcg gcg gtc cag ctc tgg gac atg tcg cgt ccg cgc aca gac gaa gac ctc aac gaa ctc ctt ggc atc acc acc atc cgc gtg acg gtc tgc gag ggc aaa aac ctg ctt cag cgc gcc aac gag ttg gtg aat cca gac gtg gtg cag gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the HSV-1 VP22 peptide having the sequence:

MTSRRSVKSGPREVPRDEYEDLYYTPSSGMASPDSPPDTSRRGALQTRSRQRG EVRFVQYDESDYALYGGSSSEDDEHPEVPRTRRPVSGAVLSGPGPARAPPPPAGSGGA GRTPTTAPRAPRTGRVATKAPAAPAAETTRGRKSAQPESAALPDAPASTAPTRSKTPA QGLARKLHFSTAPPNPDAPWTPRVAGFNKRVFCAAVGRLAAMHARMAAVQLWDMS

RPRTDEDLNELLGITTIRVTVCEGKNLLQRANELVNPDVVQDVDAATATRGRSAASRP TERPRAPARSASRPRRPVE

In still another embodiment, the fusion protein includes the C-terminal domain of the VP22 protein from, e.g., the nucleotide sequence (Nde1-EcoR1 fragment):

cat atg gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the VP22 (C-terminal domain) peptide sequence: MDVDAATATRGRSA-ASRPTERPRAPARSASRPRRPVE

In still other embodiments, the Rb inactivator is a small molecule inhibitors of Rb or p16 function.

For instance, to the extent it is relevant, the intracellular level of a native Rb inactivator, such as a cyclin or an Rb kinase, can be upregulated by inhibiting its natural turnover rate. For example, inhibitors of ubiquitin-dependent or independent degradation of the protein can be used to cause ectopic expression of protein in the sense that the concentration of the protein in the cell can be artificially elevated. Assays for detecting inhibitors of ubiquitination, e.g., which can be readily adapted for detecting inhibitors of ubiquitination of an Rb inactivator such as cyclin D1, are described in the literature, as for example US Patents 5,744,343, 5,847,094, 5,847,076, 5,834,487, 5,817,494, 5,780,454 and 5,766,927. Likewise, to the extent that other post-translational modifications, such as phosphorylation, influence protein stability, the present invention contemplates the use of inhibitors of such modifications, including, as appropriate, kinase or phosphatase inhibitors.

In still other embodiments, the subject method can be practiced with an agent, e.g., a small organic molecule that inhibits dephosphorylation of Rb, or at least formation of the hypophosphorylated for Rb (p115/hypo). Such agents may be phosphatase inhibitors or kinase activators. Rb activity is mediated by a specific protein-serine/threonine phosphatase activity. RB becomes dephosphorylated by an activated type 1 protein-serine/threonine phosphatase activity (PP1). Ludlow et al. (1993) Mol Cell Biol 13:367; and Durfee et al. (1993) Genes Dev 7:555. Indeed, addition of a specific protein-serine/threonine phosphatase inhibitor, such calyculin A, can effectively prevented formation of p115/hypo. See, An et al. (1996) Cancer Res 56:438.

As described in further detail below, the use of Rb inactivators can be used to treat cells *in vivo*, *in vitro* and *ex vivo*.

B. Inactivation of ras clock

Approach to M0 is a gradual process. In fact, normal cells do not senesce immediately upon explant, but execute a number of population doublings before their replicative potential is exhausted. During this time, the abundance of p16 increases continuously. These observations suggest that M0 arrest results from cumulative growth inhibitory signaling that eventually overwhelms a cell. We have determined that, in addition to the Rb/p16INK4a pathway, the oncoprotein *ras* provides a signal which results in a cumulative signal ultimately causing replicative senescence. Certain aspects of the ras-dependent senescence may be independent of Rb/p16INK4a pathway.

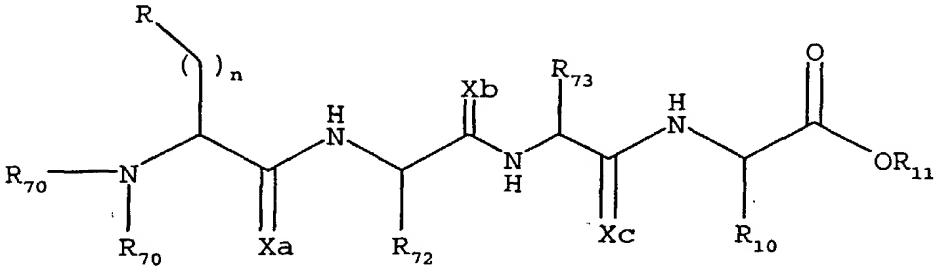
Accordingly, in addition to, or in place of utilizing an Rb inactivator described above, the subject method can utilize an agent which inhibits ras-dependent replicative senescence. In preferred embodiments, the "ras inhibitor" is an agent, preferably a small molecule inhibitor, of a ras/Raf/MKK/MAP kinase pathway.

In utilizing such inhibitors, it will likely require that the dosage of inhibitor added will be titrated to an appropriate concentration such that ras-dependent senescence is avoided but below the threshold concentration to prevent mitosis.

In certain embodiments, the agent inhibits ras activation, e.g., by inhibiting prenylation of ras or inhibiting the GTPases activity of ras.

In certain embodiments, the subject method can be practiced using a peptide or peptide-like inhibitor of a prenyl transferase activity which prenylates *ras*, preferably the inhibitor is a farnesyl transferase inhibitor.

For example, a peptidyl inhibitor of a prenyl transferase may be represented in the general formula I

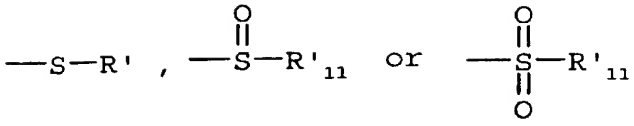


(I)

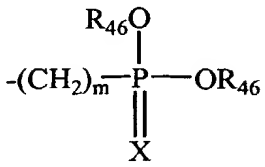
wherein

X_a, X_b and X_c each, independently, represent O or H_2;

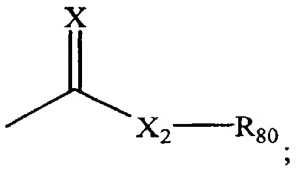
R represents



R' represents H, a lower alkyl, a lower alkenyl, an aryl,



or



;

R_7 represents an aryl, a cycloalkyl, a cycloalkenyl, or a heterocycle;

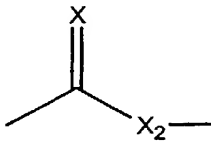
R_{10} represents a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and preferably is a sidechain of an alpha-amino acid residue or analog thereof, and even more preferably a straight chain , branched lower alkyl, aryl or arylalkyl;

R_{11} represents H, a carboxy-terminal blocking group, or a pharmaceutically acceptable salt, or

R_{10} and R_{11} taken together form a 5-7 membered lactone;

R'_{11} represents an alkyl, an alkenyl or $-(CH_2)_m-R_7$;

R_{46}, independently for each occurrence, represents hydrogen, a lower alkyl or an aryl

R_{70}, independently for each occurrence, represents H,  , a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and an alpha-carbon sidechain of an amino acid residue or analog or other amino-protecting group, or a pharmaceutically acceptable salt or

R_{70} and R taken together, or R_{70} and R_{70} taken together, form a 4 to 8 membered heterocycle;

R_{72} and R_{73}, independently for each occurrence, represents H, lower alkyl, aryl, heteroaryl, $-(CH_2)_m-R_7$ or the sidechain of an amino acid (e.g., a naturally occurring or unnatural amino acid);

R_{80} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, or

$-(CH_2)_m-R_7$;

X represents, independently for each occurrence, O or S;

X_2 represents O or S ; and

m and n, independently for each occurrence, represent zero or an integer in the range of 1 to 4.

In a preferred embodiment, the subject inhibitor is represented in Formula I, wherein X_a, X_b and X_c each represent H_2 or O, more preferably O; R represents –S-R'; R' represents H or a lower alkyl, and more preferably H; R_{72} represents a lower alkylamine, a lower alkylthiol or a lower alkyl, and more preferably CH_2NH_2, CH_2SH; R_{73} represents $-(CH_2)_m-R_7$; m=1; R_7 represents aryl, and more preferably a C6-C12 aryl, and even more preferably 2-naphthyl; R_{10} represents a lower alkyl, more preferably a branched C4-C6 lower alkyl, and even more preferably 2-methylpropyl; R_{11} represents H or lower alkyl (e.g., methyl); R_{70} for each occurrence is H.

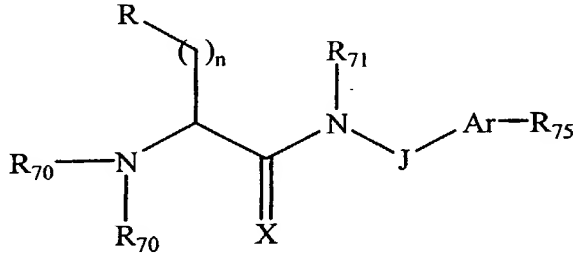
In another preferred embodiment, the subject inhibitor is represented in Formula I, wherein X_a, X_b and X_c each represent H_2 or O, more preferably X_a and X_b are H_2 and X_c is O; R represents –S-R'; R' represents H or a lower alkyl, and more preferably H; R_{72} represents a lower alkylamine, a lower alkylthiol or a lower alkyl, and more preferably isopropyl; R_{73} represents $-(CH_2)_m-R_7$; m=1; R_7 represents aryl, and more preferably a C6-C12 aryl, and even more preferably 2-naphthyl; R_{10} represents a lower alkyl, more preferably a branched C4-C6 lower alkyl, and even more preferably 2-methylpropyl; R_{11} represents H or lower alkyl (e.g., methyl); R_{70} for each occurrence is H.

In one aspect of the invention, the subject prenyl transferase inhibitors are peptidomimetics of the general formula C-A-A-X, wherein each A is, independently, an aliphatic amino acid, e.g., glycine, alanine, valine, leucine, isoleucine or an analog thereof, or A-A can represent a dipeptide equivalent spacer, C represents a cysteine or isosteric/isoelectronic equivalent thereof, and X represents any amino acid, but is preferably a methionine or serine or isosteric/isoelectronic equivalent thereof. The principal objectives in generating a peptidomimetic for use in the subject method is to increase the bioavailability of the compound and/or decrease the hydrolyzability of the peptidomimetic relative to the equivalent peptide.

To further illustrate, one class of compounds which are contemplated for use in the subject method are peptidomimetic inhibitors generated by replacing the A-A-X of the C-A-A-X tetrapeptide with a non-amino acid component while retaining the desired prenyl transferase inhibitory activity. Likewise, the cysteine residue can be replaced with an isosteric/isoelectronic equivalent, e.g., such as replacement of the sulfhydryl group with a

polar moiety such as a cyano, nitro, thiocarbamate, amino, carbamic, phosphate, thiophosphate, sulfoxide, carboximide, urea, sulfone, phosphorothioate, phosphorodithioate, thiourea, dithiocarbamate, phosphoramidodi-thioate, methylsulfonyl, phosphonate, sulfamide, phosphoramide, sulfonate, dithiocarbonate, hydroxyl, sulfate, sulfinate, sulfamate, phosphinate, carboxylate, hydroxymate, imidazole or other heterocyclic moieties. The sulfhydryl group can be functionalized, e.g., to form an S-alkyl cysteine or the corresponding sulfoxide, sulfone, sulfonate or sulfate derivatives thereof (though more preferably a sulfoxide or sulfone).

In an exemplary embodiment, the A-A-Met or A-A-Ser tripeptide is replaced with a substituted aryl or heteroaryl group which corresponds essentially in size with the tripeptide. For instance, the subject method can be performed using a farnesyltransferase inhibitor that is represented in the general formula (II):



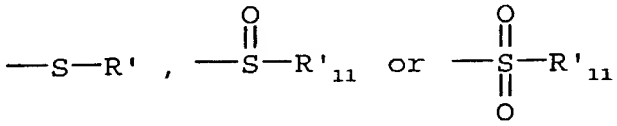
(II)

wherein

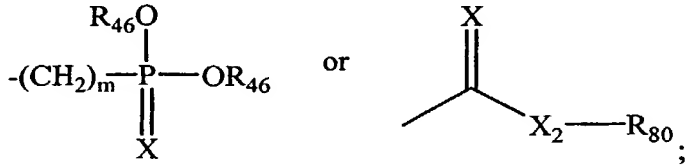
Ar represents an aryl group (e.g., substituted or unsubstituted);

J is absent (e.g., N and Ar are joined by a direct bond), or represents $-CH(R_{72})-$;

R represents



R' represents H, a lower alkyl, a lower alkenyl, an aryl,



R_7 represents an aryl, a cycloalkyl, a cycloalkenyl, or a heterocycle;

R_{10} represents a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and preferably is an alpha-carbon sidechain of an amino acid

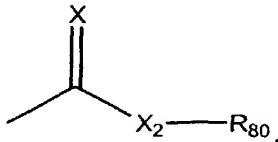
residue or analog thereof, and even more preferably a straight chain , branched lower alkyl, aryl or arylalkyl;;

R_{11} represents H, a carboxy-terminal blocking group, or a pharmaceutically acceptable salt, or

R_{10} and R_{11} taken together form a 5-7 membered lactone;

R'_{11} represents an alkyl, an alkenyl or $-(CH_2)_m-R_7$;

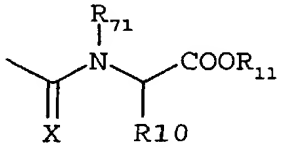
R_{46}, independently for each occurrence, represents hydrogen, a lower alkyl or an aryl;

R_{70}, independently for each occurrence, represents H,  , a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and an alpha-carbon sidechain of an amino acid residue or analog or other amino-protecting group, or a pharmaceutically acceptable salt or

R_{70} and R, or R_{70} and R_{70}, taken together form a 4 to 8 membered heterocycle;

R_{71} each independently represent H or lower alkyl;

R_{72}, independently for each occurrence, represents H, lower alkyl, aryl, heteroaryl or the sidechain of a naturally occurring amino acid;

R_{75} represents  or ;

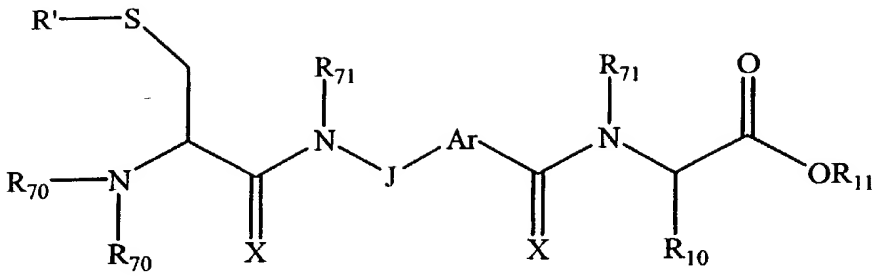
R_{80} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, or $-(CH_2)_m-R_7$;

X represents, independently for each occurrence, O, S or H_2.

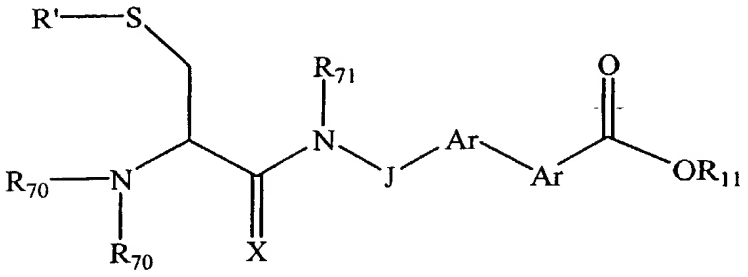
X_2 represents O or S; and

m and n, independently for each occurrence, represent zero or an integer in the range of 1 to 4.

For instance, the peptidomimetic can have a structure represented by formula IIIa or IIIb:



(IIIa)



(IIIb)

wherein

Ar, J, R', R_{70}, R_{71} and X are as defined above; and

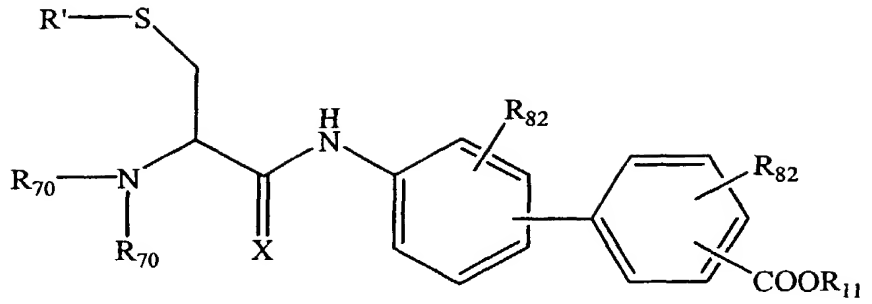
R_{10} represents a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, or an alpha-carbon sidechain of an amino acid residue or analog thereof, and is preferably a straight chain , branched lower alkyl, aryl or arylalkyl;

R_{11} represents H, a carboxy-terminal blocking group, or a pharmaceutically acceptable salt, or

R_{10} and R_{11} taken together in formula IIIa form a 5-7 membered lactone.

In preferred embodiments, Ar, for each occurrence, refers to aryl group selected from the group consisting of 5-, 6- and 7-membered monocyclic or 10-14 membered bicyclic aromatic groups that may include from zero to four heteroatoms, as for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine, pyrimidine, benzothiophene, quinoline, quinolone, and the like.

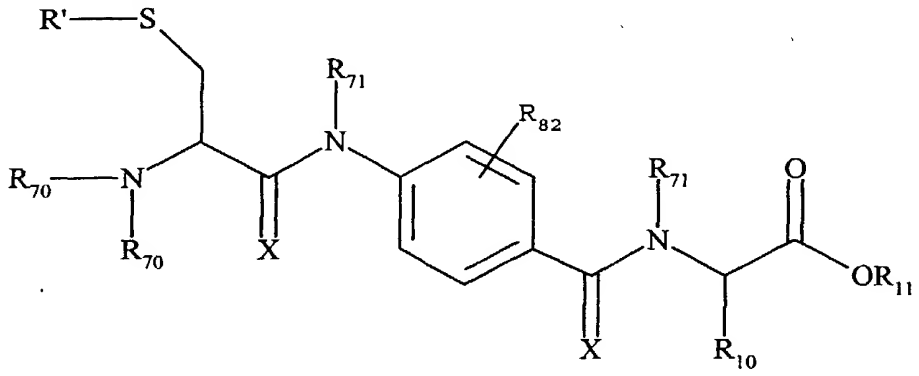
Exemplary compounds of this class can be found with the generic structures described in, *inter alia*, U.S. Patent 5,705,686 and PCT publication WO96/21456, and the class includes compounds of the general formula IVb.



IVb

wherein, X, R', R_{11}, and R_{70} are as defined above in formula IIIb, and each R_{82} is absent or represents one or more substitutions, each of which can independently be a lower alkyl, -$(CH)_2$-R_7 or $COOR_{11}$, (R_7 and R_{11} being defined above). In a preferred embodiment, the core aryl structure is a para-phenyl benzamide or meta-phenyl benzamide.

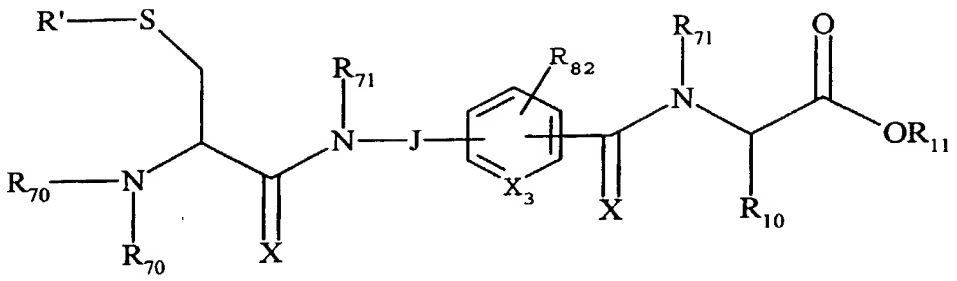
Another example of such peptidomimetics is described by Lerner et al. (1995) J Biol Chem 270:26770, as well as PCT publication WO96/21456, which each teach prenyltransferase inhibitors represented in the general formula IVa:

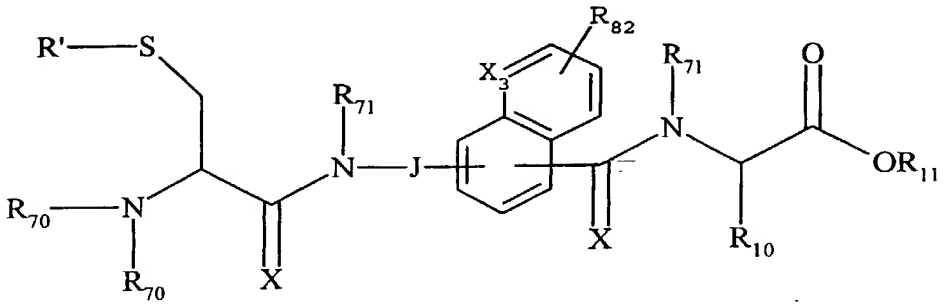


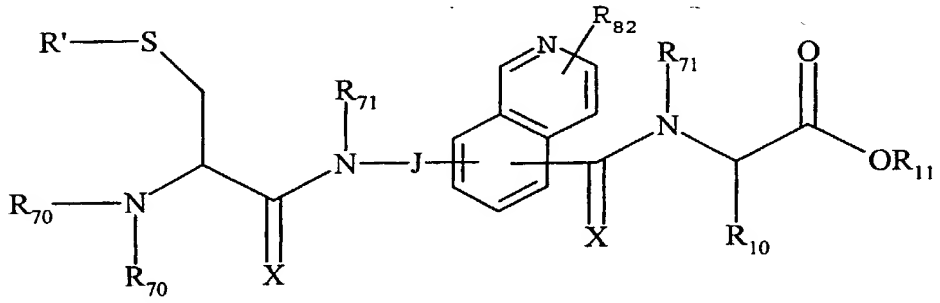
IVa

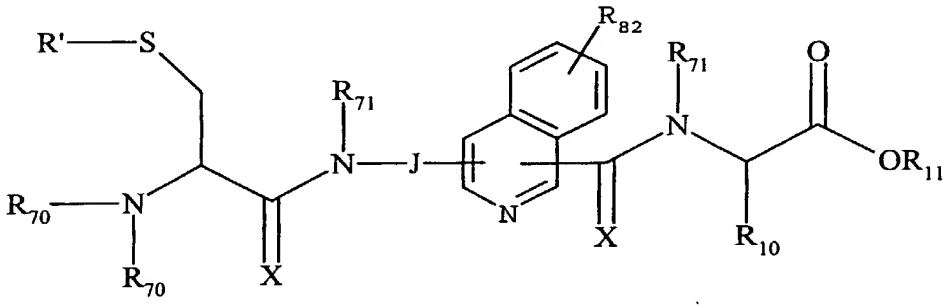
wherein, R', R_{10}, R_{11}, R_{70}, R_{71} and X are as defined above in formula IIIa, and R_{82} is absent or represent one or more substitutions, each of which can independently be a lower alkyl, -$(CH)_2$-R_7 or $COOR_{11}$, (R_7 and R_{11} being defined above).

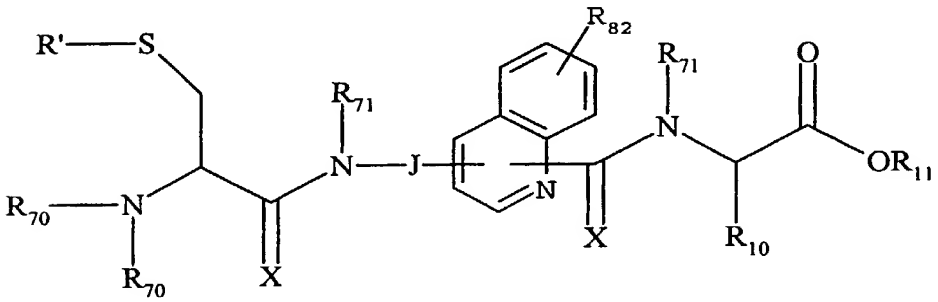
With reference to the compounds of formula IIIa, the PCT publication WO96/21456 describes a number of other aryl groups. Thus, for example, a prenyl transferase inhibitor useful as an anti agent may represented in any one of the following generic formulas:

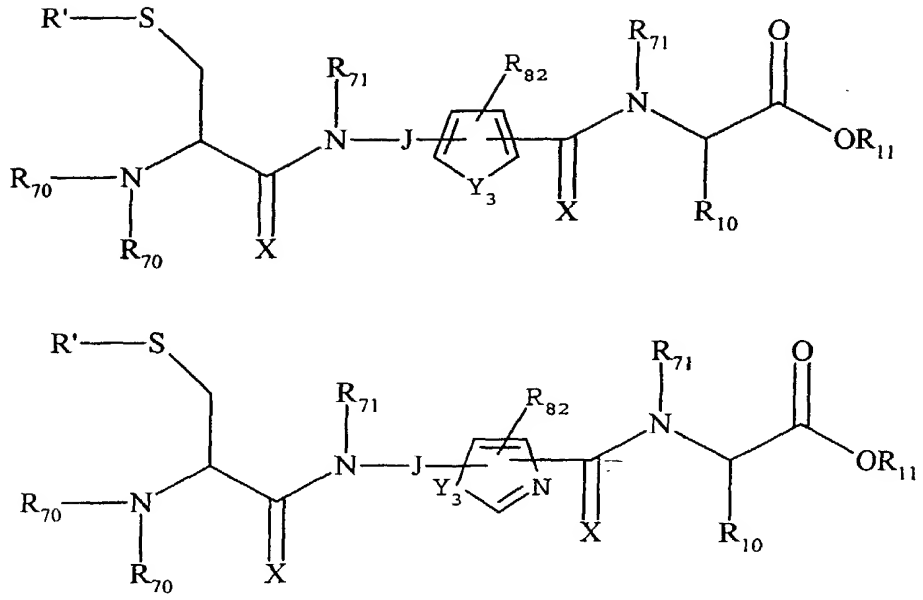






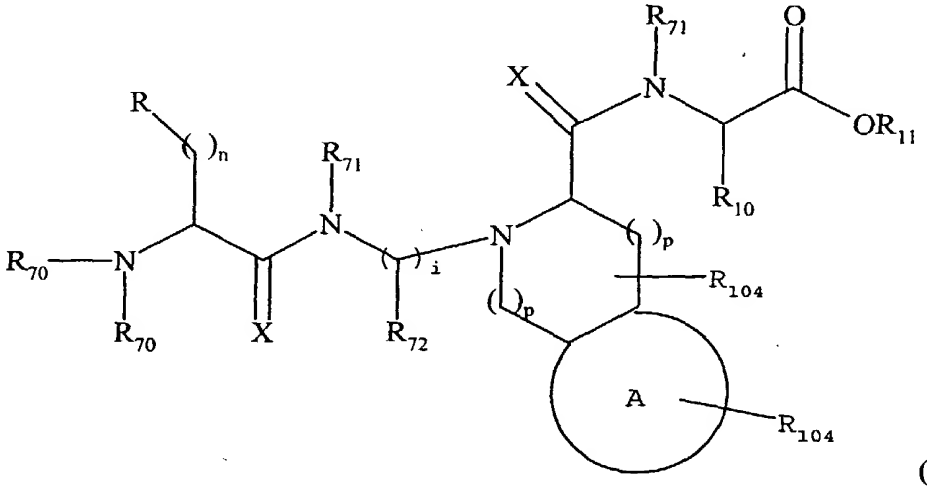






wherein R', R_{10}, R_{11}, R_{70}, R_{71}, R_{82}, J and X are as defined above, and X_3 represents C or N, and Y_3 represents O, S or NH.

In yet another embodiment, the subject method can be practiced using a compound selected from the teachings of U.S. patent 5,624,936 and of Canadian Application 2,143,588, or analogs thereof. For instance, the method of the present invention can be carried out by treatment with a prenyltransferase inhibitor represented in the general formula (V):



(V)

wherein

R, R_{10}, R_{11}, R_{70}, R_{71}, R_{72} and X are as defined above in formula I;

A represents a fused ring selected from a group consisting of a cycloalkyl, a cycloalkenyl, an aryl, and a heterocycle, wherein the fused ring A can comprise from 4 to 8

atoms in its ring structure;

R_{104} is absent or represents one or more substitutions, each independently selected from lower alkyl, aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, R_{111}-$S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, N_3, $(R_{110})_2N$- $C(NR_{110})$-, $R_{110}C(O)$-, $R_{110}OC(O)$-, $(R_{110})_2N$- or $R_{111}OC(O)NR_{110}$-, lower alkyl unsubstituted or substituted by one or more aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, $R_{111}S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, $(R_{110})_2N$-, or $R_{111}OC(O)$-NR_{110}-;

R_{110} represents hydrogen, lower alkyl, benzyl or aryl;

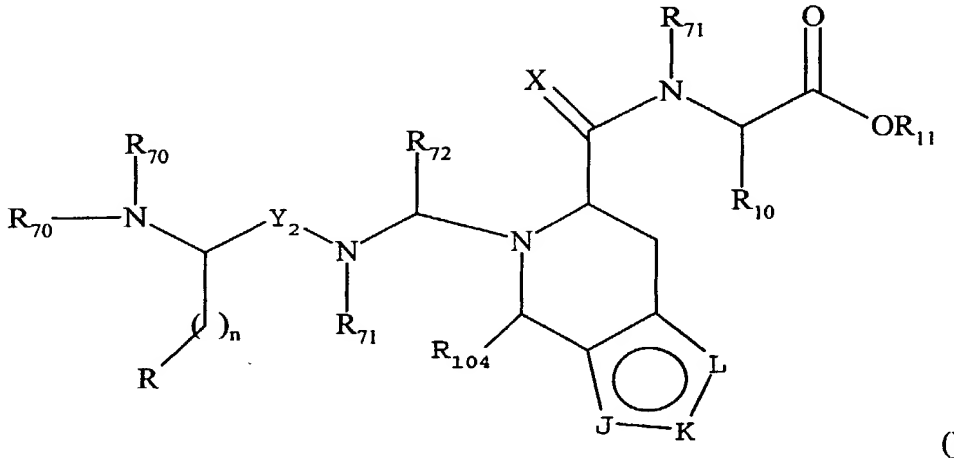
R_{111} is a lower alkyl or aryl;

i is 1, 2, or 3; and

p is, independently for each occurrence, 0, 1 or 2.

m is an integer in the range of 0 to 2.

The teachings of Canadian Application 2,143,588 are also instructive for classes of compounds which are potential inhibitors of prenyl transferases and which can be used in the present method. Thus, in another embodiment, the method of the present invention can be carried out by treatment with a compound represented in the general formula (VI):



(VI)

wherein,

R, R_{10}, R_{11}, R_{70}, R_{71}, R_{72}, R_{104}, X and n are as defined above in formula V;

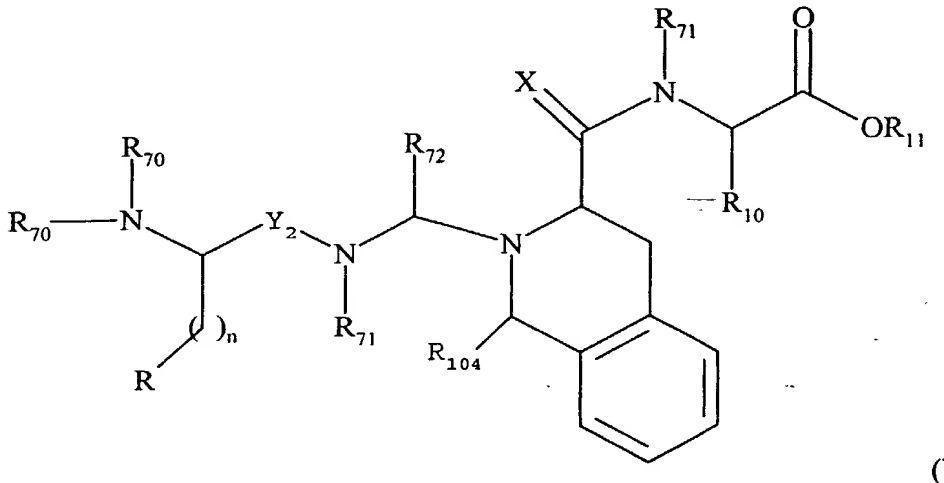
Y_2 is $-CH_2-$ or -C(O)-;

J, K and L are each independently N, NR_{105}, O, S or CR_{106}, with the proviso that only one of the three groups can be O or S, one or two of the three groups can be N or NR_{105}, and at least one must be a heteroatom to form a heteroaryl;

R_{105} represents H, lower alkyl or phenylalkyl; and

R_{106} represents H or lower alkyl.

EP publication 618,221 teaches a similar class of compounds which are potential inhibitors of prenyl transferases for use in the present method, e.g., which anti compounds may be represented in the general formula VII:



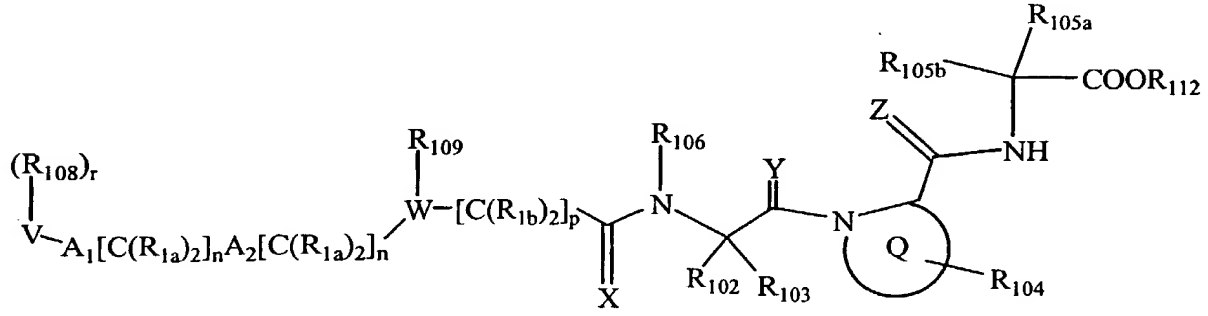
(VII)

wherein,

R, R_{10}, R_{11}, R_{70}, R_{71}, R_{72}, R_{104}, X and n are as defined above in formula V; and

Y_2 is $-CH_2-$ or -C(O)-.

The teachings of U.S. patent 5,624,936 also provide guidance for the design of other analogs which can be used in the present method. To further illustrate, the method of the present invention can be carried out by treatment with a compound represented in the general formula (VIII) (for additional structures in this class of prenyl transferase inhibitors, see: PCT application WO 97/38664):



(VIII)

wherein,

R_{1a} and R_{1b}, independently for each occurrence, are selected from hydrogen, lower alkyl, aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, R_{111}-$S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, NO_2, $(R_{110})_2N$-$C(NR_{110})$-, $R_{110}C(O)$-, $R_{110}OC(O)$-, N_3, $(R_{110})_2N$- or $R_{111}OC(O)NR_{110}$-, lower alkyl unsubstituted or substituted by one or more aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, $R_{111}S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, $(R_{110})_2N$-, or $R_{111}OC(O)$-NR_{110}-;

R_{102} and R_{103} are independently selected from a side chain of a naturally occurring amino acid, or are a lower alkyl, lower alkenyl, cycloalkyl, aryl or heterocyclic group, or

R_{102} and R_{103} taken together form a cycloalkyl, or

R_{102} along with the adjacent N form a heterocycle;

R_{104} is absent or represents one or more substitutions to Q, each independently selected from lower alkyl, aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, R_{111}-$S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, N_3, $(R_{110})_2N$- $C(NR_{110})$-, $R_{110}C(O)$-, $R_{110}OC(O)$-, $(R_{110})_2N$- or $R_{111}OC(O)NR_{110}$-, lower alkyl unsubstituted or substituted by one or more aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, $R_{111}S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, $(R_{110})_2N$-, or $R_{111}OC(O)$-NR_{110}-;

R_{105a} and R_{105b} are independently selected from a side chain of an amino acid, or otherwise a straight chain or branched lower alkyl, alkenyl, alkynyl, cycloalkyl, aryl or heterocycle;

R_{106} represents hydrogen or a lower alkyl;

R_{108} and R_{109} represent, independently, hydrogen, alkyl, aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, halogen, $R_{110}O$-, R_{111}-$S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, N_3, $(R_{110})_2N$-$C(NR_{110})$-, $R_{110}C(O)$-, $R_{110}OC(O)$-, $(R_{110})_2N$- or $R_{111}OC(O)NR_{110}$-, lower alkyl unsubstituted or substituted by one or more aryl, heterocycle, cycloalkyl, alkenyl, alkynyl, $R_{110}O$-, $R_{111}S(O)_m$-, $R_{110}C(O)NR_{110}$-, CN, $(R_{110})_2N$-, or $R_{111}OC(O)$-NR_{110};

R_{110} represents hydrogen, lower alkyl, benzyl and aryl;

R_{111} is a lower alkyl or aryl;

Q is a substituted or unsubstituted nitrogen-containing bicyclic ring system;

V represents hydrogen, lower alkyl, lower alkenyl, aryl or heterocycle;

W is a heterocycle;

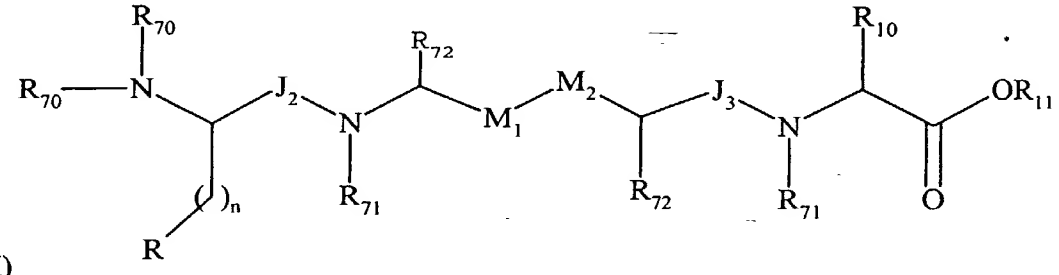
X, Y and Z are independently O, S or H_2;

m is 0, 1 or 2;

n and p are, independently, 0, 1, 2, 3 or 4; and

r is an integer in the range of 0-5.

US Patent 5,470,832 and PCT publication WO95/20396 provide insight into still other embodiments of compounds wherein the backbone of a peptide inhibitor is replaced with a non-hydrolyzable analog thereof. Accordingly, in certain embodiments of the subject method, the prenyl transferase inhibitor can be a compound represented in the general formula IX



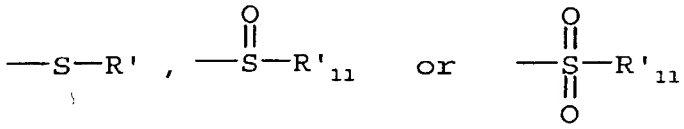
(IX)

wherein

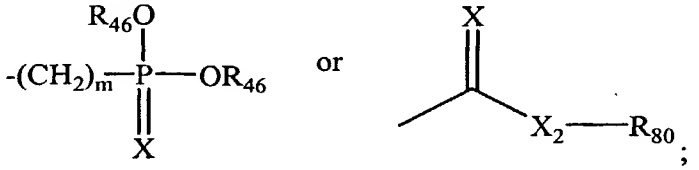
M_1-M_2 represents -CH_2-O- or -CH=CH-;

J_2 and J_3 each represent -CH_2- or -C(X)-;

R represents



R' represents H, a lower alkyl, a lower alkenyl, an aryl,

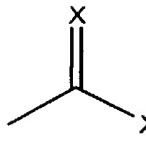


R_7 represents an aryl, a cycloalkyl, a cycloalkenyl, or a heterocycle;

R_{11} represents H, a carboxy-terminal blocking group, or a pharmaceutically acceptable salt;

R'_{11} represents an alkyl, an alkenyl or -$(CH_2)_m$-R_7

R_{46}, independently for each occurrence, represents hydrogen, a lower alkyl or an aryl



R_{70}, independently for each occurrence, represents H, , a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and an alpha-carbon sidechain of an amino acid residue or analog or other amino-protecting group, or a pharmaceutically acceptable salt or

R_{70} and R taken together form a 4 to 8 membered heterocycle;

R_{71} represents H or a lower alkyl;

R_{72}, independently for each occurrence, represents H, lower alkyl, aryl, heteroaryl or the sidechain of a naturally occurring amino acid;

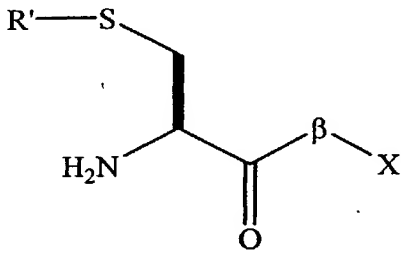
R_{80} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, or $-(CH_2)_m-R_7$;

X represents, independently for each occurrence, O or S;

X_2 represents O or S; and

m and n, independently for each occurrence, represent zero or an integer in the range of 1 to 4 inclusive.

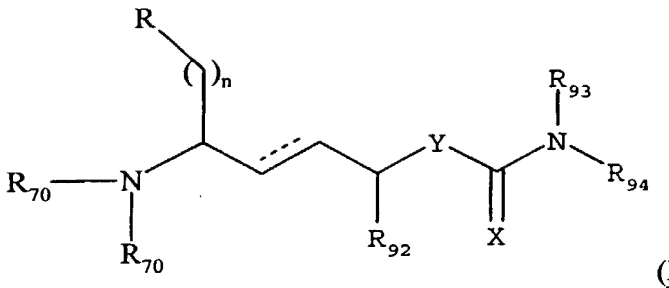
In other embodiments, the subject compounds may be selected from the generic structures described in U.S. Patent 5,602,098, and may be represented in the general formula X:



(X)

wherein R' is defined above; X is a leucine residue, or analog thereof; and β is a residue of *ortho-*, *meta-*, or *para*-aminobenzoic acid, or a residue of an aminoalkylbenzoic acid.

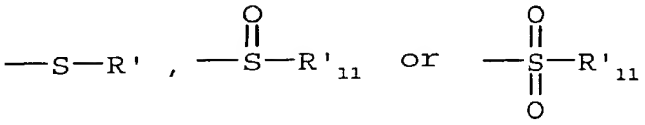
Inhibitors of prenyl transferases may also be selected from amongst the class of compounds disclosed in the PCT publication WO95/25086, e.g., represented in the general formula (XI):



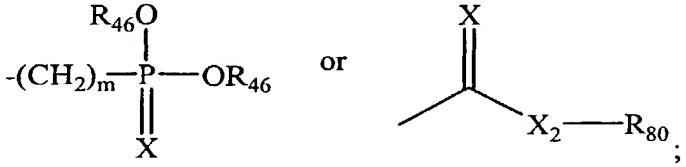
(XI)

wherein

R represents



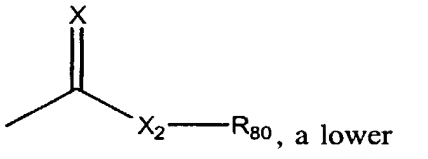
R' represents H, a lower alkyl, a lower alkenyl, an aryl,



R_7 represents an aryl, a cycloalkyl, a cycloalkenyl, or a heterocycle;

R'_{11} represents an alkyl, an alkenyl or $-(CH_2)_m-R_7$

R_{46}, independently for each occurrence, represents hydrogen, a lower alkyl or an aryl

R_{70}, independently for each occurrence, represents H,  , a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and an alpha-carbon sidechain of an amino acid residue or analog or other amino-protecting group, or a pharmaceutically acceptable salt or

R_{70} and R taken together form a 4 to 8 membered heterocycle;

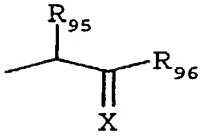
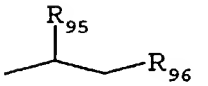
R_{92} represents H, lower alkyl, aryl, heteroaryl or the sidechain of an amino acid;

R_{80} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, or $-(CH_2)_m-R_7$;

X represents, independently for each occurrence, O or S;

X_2 represents O or S; and

R_{93} represents H, lower alkyl, aryl or heteroaryl;

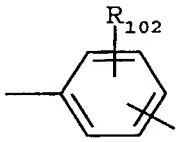
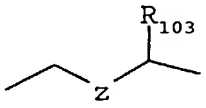
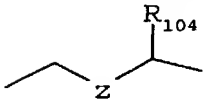
R_{94} represents a cycloalkyl, a heterocycle, an aryl,  ,  , $-CH_2-R_{95}$, or any other amino-protecting group;

R_{95} represents a lower alkyl, a heterocycle, an aryl, a lower alkoxyl, $-(CH_2)_n-A-(CH_2)_m$-lower alkyl (wherein A is O, S, SO or SO_2), or any other side chain of a naturally occurring amino acid;

R_{96} represents H, $-NH_2$, -NHOH, heterocycle, aryl, $-N(R_{97})_2$, $-OR_{98}$, $-N(R_{97})OR_{98}$, $-NHOR_{98}$, or any other carboxyl-protecting group;

R_{97}, independently for each occurrence, represents a lower alkyl, a heterocycle, an alkyloxycarbonyl, an aryl or any other amino-protecting group;

R_{98}, independently for each occurrence, represents H, a lower alkyl, an acyloxyalkyl, alkyloxyalkyl, alkyloxycarbonyl or another hyrdoxyl- or carbonyl-protecting group;

Y is selected from the group consisting of  ,  , and 

R_{102} is absent or represents one or more substitutions independently being a halogen, -OH, a lower alkyl, a lower alkenyl, a lower alkynyl, an alkoxyl, an acyloxyl, an acyl, an aryl, a heterocycle, an alkylsulfonyloxyl, a haloalkylsulfonyloxyl, an arylsulfonyloxyl, or an aryloxyl;

R_{103} represents H, a lower alkyl, an aryl, or a heterocycle;

R_{104} represents H, a lower alkyl, an aryl, or a heterocycle;

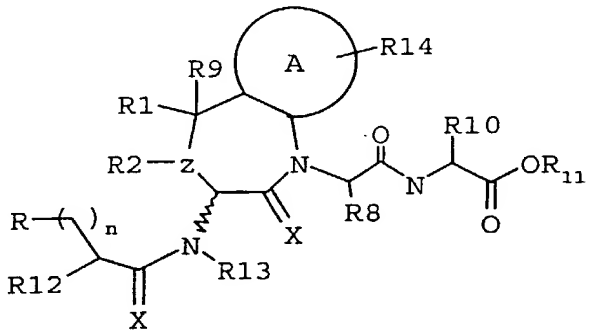
Z represents O, S, SO, SO_2 or an amine;

m and n, independently for each occurrence, represent zero or an integer in the range of 1 to 4 inclusive.

In another embodiment, the prenyl transferase inhibitor is an azepine-derived peptidomimetic represented by the general formula α-amino-N-[1-(2-Met-2-oxoethyl)-1-azepin-3-yl]-Cys (Formula XII), wherein Cys represents a cysteine or a cysteine analog which

is carboxy-terminally linked with a 3-amino moiety of an azepine, and Met represents a methionine or methionine analog amino-terminally linked through a peptide bond with the 2-oxoethyl moiety of the azepine. The azepine core mimics a dipeptidyl amide backbone, and the Cys, azepine, and Met (or Ser) moieties together form a peptidyl analog of the general formula Cys-Xaa-Xaa-Met or Cys-Xaa-Xaa-Ser. In certain embodiments of the present invention, the Cys moiety can further include an additional amino acid residue or peptide, linked in a peptidyl bond to the N-terminus of the methionine in order to further extend the peptidomimetic at the amino terminus.

In an exemplary embodiment, the peptidyl-azepine is represented by Formula XIII (numerous examples of prenyl transferase inhibitors of this general structural class are described in US Patent 5,532,359):

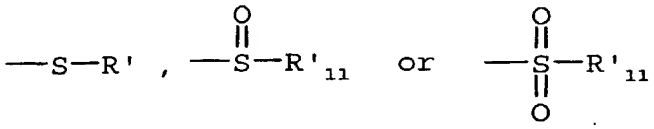


(XIII)

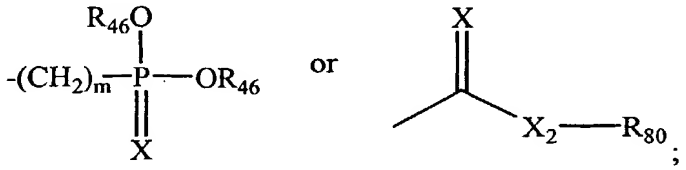
wherein

A represents a fused ring selected from a group consisting of a cycloalkyl, a cycloalkenyl, an aryl, and a heterocyclic ring, wherein the fused ring A can comprise from 4 to 8 atoms in its ring structure;

R represents



R' represents H, a lower alkyl, a lower alkenyl, an aryl,



R_1, R_2, R_8 and R_{10} each independently represent hydrogen, halogen, alkyl, alkenyl, alkynyl, hydroxyl, alkoxyl, silyloxy, amino, nitro, sulfhydryl, alkylthio, imine, amide, phosphoryl,

phosphonate, phosphine, carbonyl, carboxyl, carboxamide, anhydride, silyl, thioalkyl, alkylsulfonyl, arylsulfonyl, selenoalkyl, ketone, aldehyde, ester, heteroalkyl, nitrile, guanidine, amidine, acetal, ketal, amine oxide, aryl, heteroaryl, azide, aziridine, carbamate, epoxide, hydroxamic acid, imide, oxime, sulfonamide, thioamide, thiocarbamate, urea, thiourea, or -$(CH_2)_m$-R_7;

R_4 and R_5 each independently represent hydrogen, lower alkyl, lower alkenyl, -$(CH_2)_m$-R_7, -C(O)-lower alkyl, -C(O)-lower alkenyl, -C(O)-$(CH_2)_m$-R_7, or a pharmaceutically acceptable salt forming ion,

or R_4 and R_5 taken together with the N atom to which they are attached complete a heterocyclic ring having from 4 to 8 atoms in the ring structure;

R_7 represents an aryl, a cycloalkyl, a cycloalkenyl, or a heterocycle;

R_9 is a hydrogen or a lower alkyl;

R_{11} represents H, a carboxy-terminal blocking group, or a pharmaceutically acceptable salt;

R'_{11} represents an alkyl, an alkenyl or -$(CH_2)_m$-R_7;

R_{12} represents N(-R_4)R_5;

R_{13} represents hydrogen, or a lower alkyl;

R_{14} is absent or represents one or more substitutions with halogens, lower alkyls, lower alkoxyls, lower alkylthiols, -NO_2, -CF_3, -CN, and -OH;

R_{46}, independently for each occurrence, represents hydrogen, a lower alkyl or an aryl;

R_{80} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, or -$(CH_2)_m$-R_7;

X and X_2, for each occurrence, represents O or S;

Z represents C or N; and

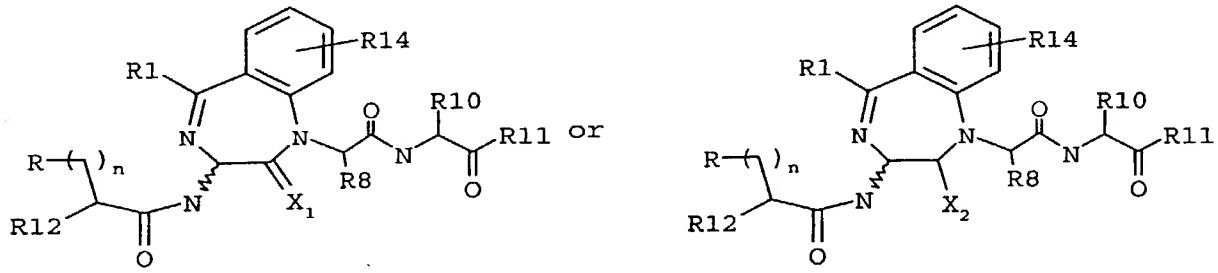
n is zero or an integer in the range of 1 to 6 inclusive; and m is an integer in the range of 0 to 6 inclusive .

In preferred embodiments, the fused ring A is selected from a group consisting of benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyrrolidine, pyridine, pyrazine, pyridazine and pyrimidine, and the like. The fused ring A can be substituted, for example, by any of a halogen, a lower alkyl, a lower alkoxy, a lower alkylthio, -NO_2, -CF_3, -CN, and -OH. Though it will be understood that in some instances it may be undesirable to have a substituent, such as a halogen or a nitro group, in the 7 position

(particularly wherein A is a benzene ring) as such substituents are generally required for sedative-hypnotic activity in other benzodiazepines, such as diazepam or nitrazepam.

Likewise, in preferred, yet optional, embodiments, R_1 is particularly selected from a group consisting of $-(CH_2)_m$-phenyl, $-(CH_2)_n$-S-$(CH_2)_m$-phenyl, $-(CH_2)_n$-O-$(CH_2)_m$-phenyl, $-(CH_2)_m$-pyridyl, $-(CH_2)_n$-S-$(CH_2)_m$-pyridyl, and $-(CH_2)_n$-O-$(CH_2)_m$-pyridyl. Additionally, each of the benzyl and pyridyl moieties can be substituted at one or more positions with a halogen, a lower alkyl, a lower alkoxy, a lower alkylthio, $-NO_2$, $-CF_3$, -CN, and -OH. The choice of R_1, as well as the other substituents of the azepine peptidomimetic, can effect the solubility, as well as membrane partioning of the subject peptidomimetics. For instance, as a result of their pyridyl-substituted nature, pyridyl containing R_1 substituents can exhibit a greater water solubility than the analogous phenyl-substituted azepines.

In an exemplary embodiment, the peptidomimetic of the present invention is a benzodiazepine represented by the general formula XIV (for specific examples of compounds of this formula, and representative synthetic schemes, see: *inter alia* US Patent 5,580,979):



(XIV)

wherein

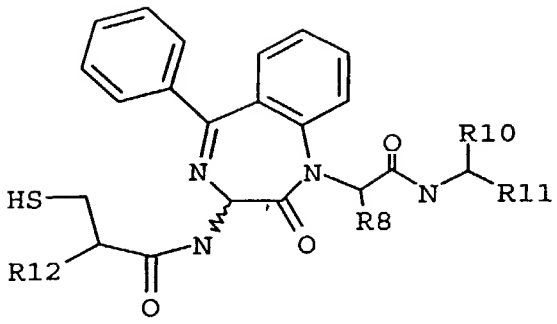
R, R_1, R_8, R_{10}, R_{11}, R_{12}, R_{14} are as defined above in formula XIII;

X_1 represents O or S; and

X_2 represents hydrogen, a lower alkyl, $-(CH_2)_m$-OH, $-(CH_2)_m$-O-lower alkyl, a carboxyl, an amide, a nitrosyl, a sulfhydryl, a sulfonyl, or a sulfonamide;

n is zero or an integer in the range of 1 to 6 inclusive; and m is an integer in the range of 1 to 6 inclusive

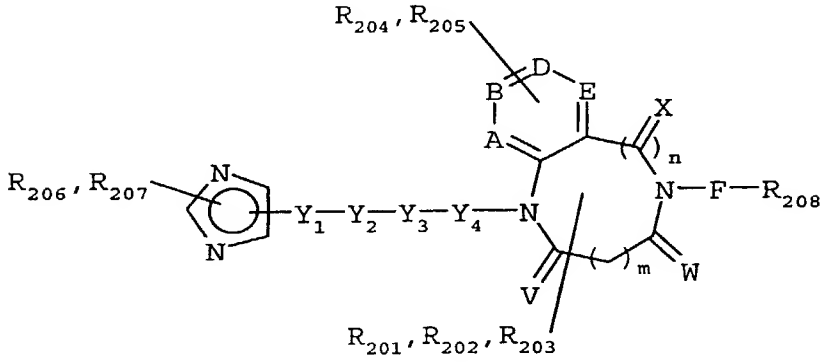
For instance, the peptidomimetic can be a 5-phenyl substituted 1,4-diazepine represented by the general formula XV:



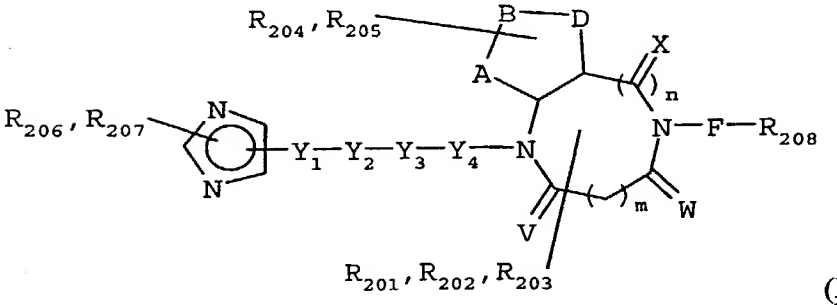
(XV)

wherein R_8, R_{10}, R_{11}, R_{12} are as defined above in formula XIII.

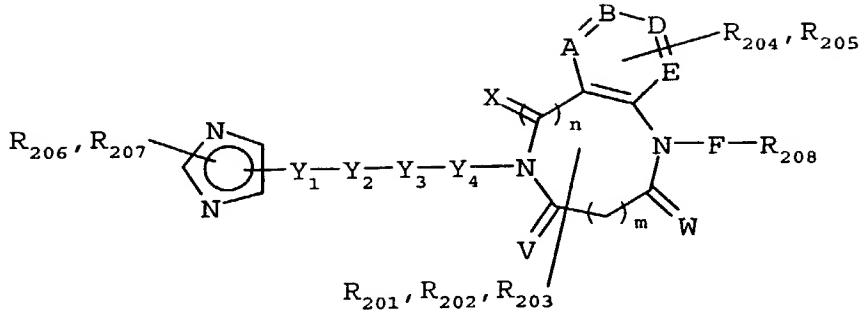
Another class of azepine-derived mimetics from which a prenyl transferase inhibitor can be selected are described in PCT publication WO97/30992, e.g., the inhibitor may be represented in one of the general formulas XVI, XVII, XVIII, XIX:



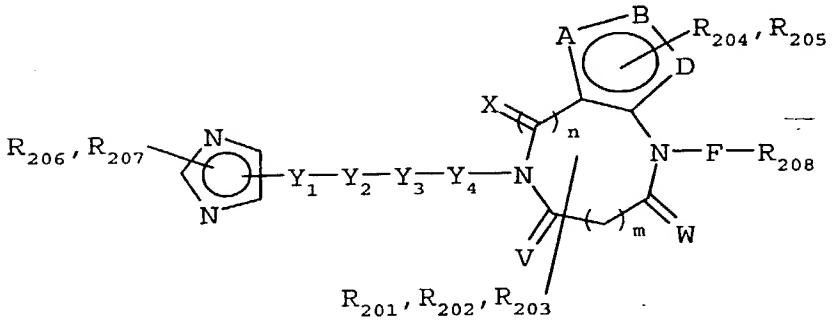
(XVI)



(XVII)



(XVIII)



(XIX)

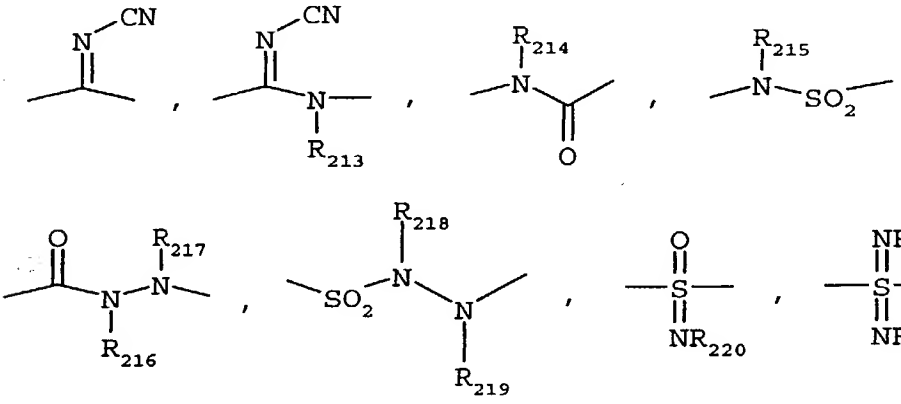
wherein

m and n are, independently, 0 or 1;

p is 0, 1 or 2;

V, W and X are selected from the group consisting of O, H_2, R_{201}, R_{202} or R_{203};

F and Y_4 are selected from the group consisting of CHR_{209}, SO_2, SO_3, CO, CO_2, O, NR_{210}, SO_2, SO_3, CO, CO_2, O, NR_{210}, SO_2NR_{211}, $CONR_{212}$,



or F may be absent;

R_{206}, R_{207}, R_{209}, R_{210}, R_{211}, R_{212}, R_{213}, R_{214}, R_{215}, R_{216}, R_{217}, R_{218}, R_{219}, R_{220}, R_{221}, R_{222}, R_{224}, R_{225}, R_{226}, R_{227}, R_{228}, R_{229}, R_{230}, R_{231}, R_{232}, R_{233}, R_{234}, R_{235}, R_{236}, R_{237}, and R_{238} are, independently, selected from the group consisting of H, lower alkyl or

aryl;

R_{204} and R_{205} are selected from the group consisting of H, halogens, nitro, cyano, and U-R_{223};

U is selected from the group consisting of S, O, NR_{224}, CO, SO, SO_2, CO_2, $NR_{25}CO_2$, $NR_{26}CNR_{27}$, $NR_{28}SO_2$, $NR_{29}SO_2NR_{30}$, SO_2NR_{31}, $NR_{32}CO$, $CCONR_{33}$, PO_2R_{34}, PO_3R_{35} or U is absent;

R_{201}, R_{202}, R_{203} are absent or, each independently, selected from the group consisting of alkyls, alkoxycarbonyl, alkenyl, alkynyl, aralkyl, cycloalkyl, aryl, heterocycle, cyano, carboxy and carbamyl, or cases where there are two substituents on a single nitrogen, selected from the group consisting of alkyl, aryl or araalkyl, or

any two of the R_{201}, R_{202} and R_{203} taken together form a cycloalkyl or heterocycle;

R_{208} and R_{223} are selected from the group consisting of H, alkyls, alkenyls, alkynyls, aralkyls, cycloalkyls, aryls and heterocycles;

Y_1, Y_2, and Y_3 are, independently, absent or selected from the group consisting of $-CH_2-$, -C(O)- and $-CH(CH_2)_pQ-$;

Q is NR_{236}, R_{237}, OR_{238} or CN; and

A, B, D and E are C, O, S or N,

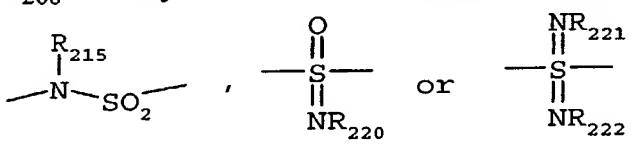
with the provisos that

(i) when m is zero, then V and W are not both oxygens; or

(ii) W and X together can be oxygen only if F is either absent, O, NR_{210}, CHR_{209}, $-N(R_{214})-C(O)-$ or $-N(R_{215})-SO_2-$ in formulas XVII and XVIII, and V and X together with can be oxygen only if F is O, NR_{210}, CHR_{209}, $-N(R_{214})-C(O)-$ or $-N(R_{215})-SO_2-$ in formulas XIX and XX; or

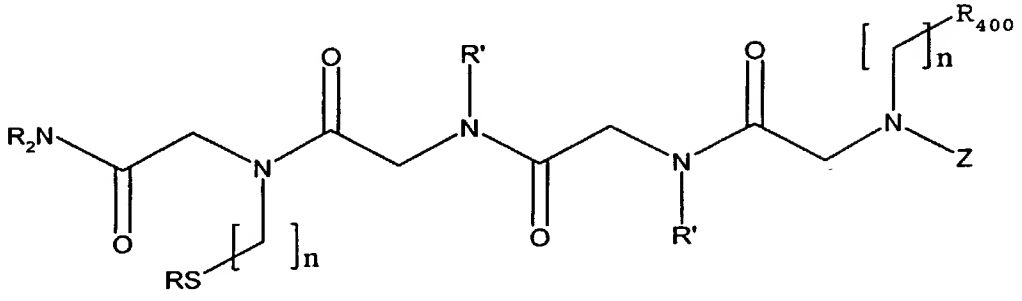
(iii) R_{223} may be H_2 except when U is SO, SO_2, $NR_{225}CO_2$, or $NR_{228}SO_2$; or

(iv) R_{208} may be H except when F is SO_2, CO_2,



In still other embodiments, the subject prenyltransferase inhibitor is represented by one of the following formulas. First, the subject compounds may be *retro N*-alkyl oligoglycine peptoids (Simon et al. *Proc. Natl. Acad. Sci., USA* **1992**, *89*, 9367; Zuckermann et al. *J. Med.*

Chem. **1994**, *37*, 2678), represented by Formula XX:



XX

wherein

R represents, independently for each occurrence, H, Me, lower alkyl, aryl, aralkyl, heteroalkyl, or heteroaryl;

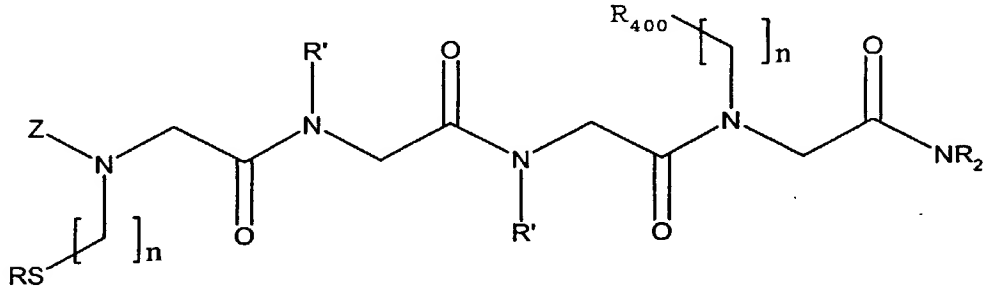
R' represents, independently for each occurrence, Me, lower alkyl, aryl, aralkyl, heteroalkyl, or heteroaryl;

R_{400} represents S-R or O-R, where R is defined above;

Z represents H, Me, lower alkyl, aryl, aralkyl, heteroalkyl, heteroaryl, acyl, sulfonyl, -C(O)OR, or -$C(O)N(R)_2$; and

n represents, independently for each occurrence, an integer in the range 1 to 3 inclusive.

Second, the subject compounds may be *N*-alkyl oligoglycine peptoids, represented by Formula XXI:



XXI

wherein

R represents, independently for each occurrence, H, Me, lower alkyl, aryl, aralkyl, heteroalkyl, or heteroaryl;

R' represents, independently for each occurrence, Me, lower alkyl, aryl, aralkyl, heteroalkyl, or heteroaryl;

R_{400} represents S-R or O-R, where R is defined above;

Z represents H, Me, lower alkyl, aryl, aralkyl, heteroalkyl, heteroaryl, acyl, sulfonyl, -C(O)OR, or -$C(O)N(R)_2$; and

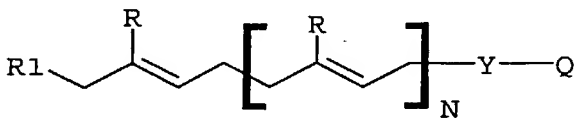
n represents, independently for each occurrence, an integer in the range 1 to 3 inclusive.

As noted above, certain peptidomimetics of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including *cis*- and *trans*-isomers, *R*- and *S*-enantiomers, diastereomers, D-isomers, L-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomer. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts may be formed with an appropriate optically-active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

In certain embodiments, the prenyl transferase inhibitors of the subject method are non-peptide inhibitors of prenyl transferase. For example, the methods of the present invention can be carried out with analogs of prenyldiphosphates, particularly farnesyl diphosphate. Such inhibitors include acyclic terpenes. Terpenes are organic compounds constructed of multiples of 2-methyl-1,3-butadiene. The inhibitors of the present invention can be analogs of monoterpenes (those containing two isoprene units, such as myrcenyl moieties), sesquiterpenes (those containing three such units, such as farnesyl moieties) or diterpenes (those containing four isoprene subunits, such as geranylgeranyl moieties).

In an illustrative embodiment, the terpene-derived prenyl transferase inhibitor is represented in the general formula (XXII):



XXII

wherein

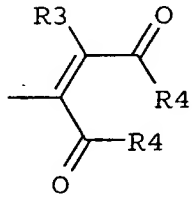
R, independently for each occurrence, represents a halogen or lower alkyl;

R_1 represents -H, -OH, -O-alkyl, -O-aryl, -O-C(O)-H, -O-C(O)-alkyl, or -O-C(O)-aryl;

Y represents a bond (i.e. is absent) or -S-, -O-, $-(CH_2)_m-$,

Q represents $-C_1-C_6$alkyl-R_2, -C(O)-R_2, -NH-$(CH_2)_n$-R_2, -NH-C(O)-$(CH_2)_n$-R_2, -C(O)-NH$(CH_2)_n$-R_2;

R_2 represents a hydrogen, a lower alkyl, or a phosphate or bisphosphate or analog thereof such as sulfate, sulfonate, sulfamoyl, sulfinyl, sulfoxyl, sulfinate, phosphoryl, phosphorothioate, phosphoramidite, phosphonamidite or boronate;

or Y and Q taken together represent  , R_3 represents a hydrogen or lower alkyl, and R_4, independently for each occurrence, represents a hydrogen, lower alkyl, -OH, -O-lower alkyl, or a carboxyl blocking group;

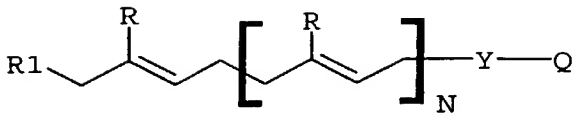
m, independently for each occurrence, is an integer in range of 1 to 6 inclusive;

n, independently for each occurrence, is zero or an integer in range of 1 to 6 inclusive; and

N is an integer in the range of 1 to 3 inclusive (though preferably 2).

For example, the art describes, in the context of inhibition of mammalian FPTases or prenyl transferases, a variety of analogs of isoprenyl diphosphates, e.g., wherein the biologically labile diphosphate moiety is replaced with a group that is a stable isostere. The various compounds described in the art, and certain equivalents that may be evident therefrom, can be tested for inhibition of cell growth either directly, or by first assessing the compounds in such high throughput, cell-free assays as described herein.

For instance, Macchia et al. (1996) J Med Chem 39:1352 describes non-peptidic inhibitors of mammalian prenyl transferase activity. The compounds described by Macchia et al. include those which are represented in the general formula XXII (as above)



wherein

N=2;

each R represents a methyl;

R_1 represents hydrogen;

Y represents -O-;

Q represents $C(O)-NH(CH_2)_n-R_2$ or $-NH-C(O)-(CH_2)_n-R_2$; and

R_2 represents a sulfamoyl, phosphoryl or phosphorylalkyl.

The Balsamo PCT publication WO97/19091 describes other prenyl transferase inhibitors which may be useful in the subject method. For instance, the compounds described in this application are also represented in general formula XXII above, wherein

Y represents $-CH_2-X-A-$, CH_2-CH_2, or -CH(OH)-;

X represents -ONH-, -O-NH-C(O)-, $-OCH_2C(O)-$, $OCH_2P(O)(OH)-$, -NHC(O)-, $-NCH_3C(O)-$, $-O-SO_2-$, or $-NHSO_2-$;

A represents $-C(R')(R'')-$, $-C(R')HCH_2-$, NH when X= $-OSO_2-$, or $-NHSO_2-$;

B represents -OC(O)-, -O-, -ONHC(O)-, -NHC(O)-, or $-NCH_3C(O)-$; and

R', R" each independently represent H, CH_3, or CH_2CH_3;

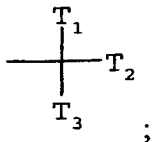
The Rando PCT publication WO 94/01126 teaches yet another class of prenyl transferase inhibitors, including those represented in the general formula:

$W-Y-CH_2-Q$

wherein

W represents farnesyl, geranylgeranyl, substituted farnesyl, or substituted geranylgeranyl;

Y represents -S-, -O-, $-CH_2-$,



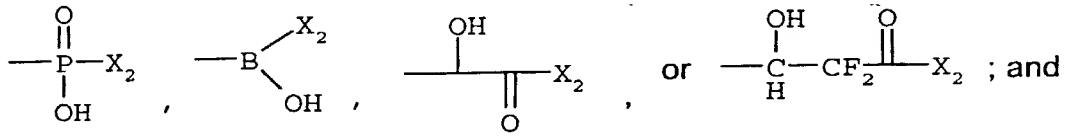
Q represents ;

T_1 represents H, F, or $-(CH_2)_n-X_1$;

T_2 is $-NHCOCH_3$, $-NH-(CH2)_n-X_1$, $-NHC(O)-OC(CH_3)_3$, or an oligopeptide of 20 or fewer amino acids, linked to the carbon via the N terminal nitrogen;

X_1 represents -SH, -COOH, $CONH_2$;

T_3 represents $-C(O)-X_2$, -CH(O), $-C(O)-CF_3$, $-C(O)-CF_2-X_2$, $-CH(OH)-(CH_2)_n-C(O)-X_2$, $-CH_2-X_2$, $-CF_2-X_2$,

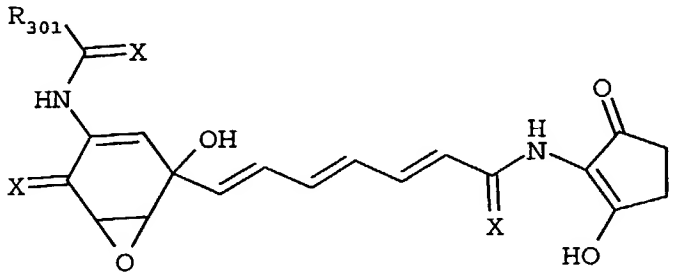


; and

X_2 represents a peptide of 20 or fewer amino acids, linked to the carbon via the N terminal nitrogen.

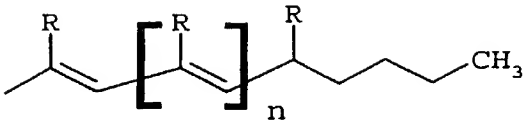
In preferred embodiments, Q is a peptide or peptidyl moiety which resembles the substrate of a prenyl transferase, e.g., a sequence from a Rho1-like phosphatase which includes the prenyl transferase recognition sequence.

Hara et al. (1993) PNAS 90:2281 describes a generic class of non-peptidyl inhibitors of FTase inhibitors which could be screened for activity (and selectivity) against prenyl transferases. Thus, in another embodiment of the present method the anti agent may be represented in the general formula:



wherein

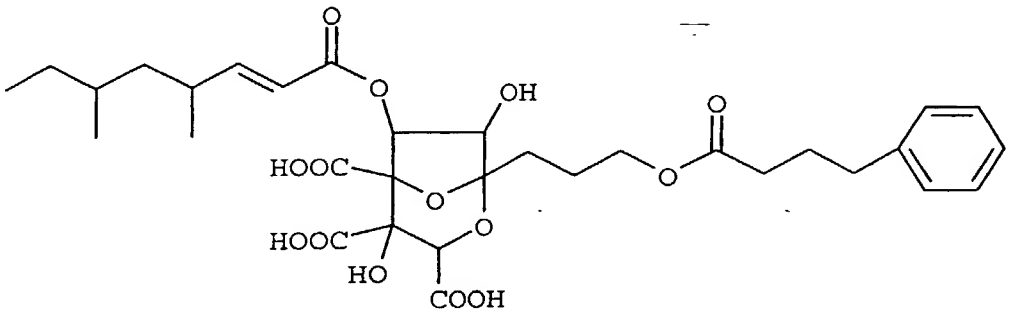
X is O or S;



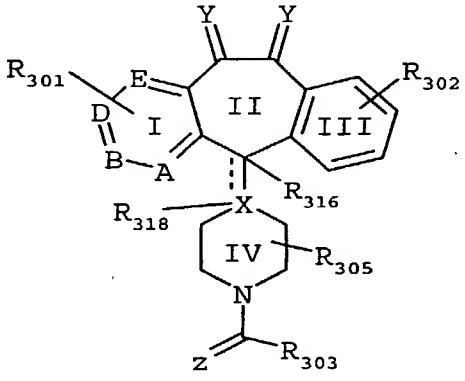
R_{301} represents ;

and n is 0, 1 or 2.

Prenyl transferase inhibitors which are useful in the method of the present invention may also be found in the compounds described in the PCT publication WO92/20336, e.g., which are similar to the structure:



In still other embodiments of the subject method, the inhibitor of the ras prenyl transferase is a small organic molecule which is neither peptidyl or prenyl in nature. For example, U.S. Patent 5,721,236 describes tricyclic carbamate compounds and the like as inhibitors of mammalian FTase activities. It is contemplated herein that within the generic class of compounds disclosed in that patent there exist inhibitors selective for a prenyl transferase, e.g., represented in the general formula:



wherein,

A, B, D and E independently represent C or N or NR_{309};

Y, independently for each occurrence, represents O or H_2;

X represents N or C;

Z represents O or S;

R_{301} is absent, or represents one or more substitutions of the ring I, each independently selected from halogens, $-CF_3$, $-OR_{310}$, $-COR_{310}$, $-SR_{310}$, $-N(R_{310})_2$, $-NO_2$, $-C(O)R_{310}$, $-CO_2R_{310}$, $-0C0R_{310}$, benzotriazol-1-yloxy, CN, alkynyl, alkenyl or alkyl;

R_{302} is absent, or represents one or more substitutions of the ring III, each independently selected from halogens, $-CF_3$, $-OR_{310}$, $-COR_{310}$, $-SR_{310}$, $-N(R_{310})_2$, $-NO_2$, $-C(O)R_{310}$, $-CO_2R_{310}$, $-0C0R_{310}$, benzotriazol-1-yloxy, CN, alkynyl, alkenyl or alkyl;

R_{303} represents $-SR_{310}$, $-OR_{310}$, $-N(R_{310})_2$ or $-(CH_2)_mR_{310}$;

R_{305} is absent, or represents one or more substitutions of the ring IV, each independently selected from halogens, $-CF_3$, alkyl, or aryl;

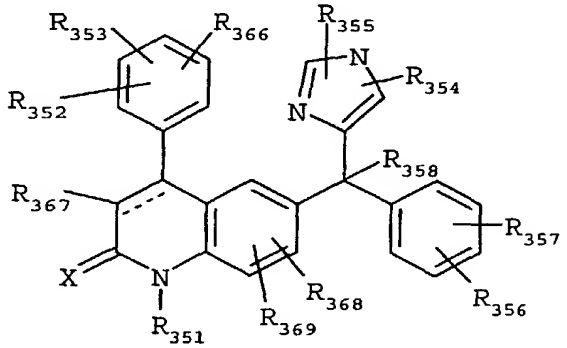
R_{310}, independently for each occurrence, represents H, alkyl, cycloalkyl, aryl or aralkyl;

R_{316} and R_{318} each independently represent H or F when the bond to X is a single bond and X is C, or R_{318} is absent when X is N, or both R_{316} and R_{318} are absent when the bond to X is a double bond (and X is C);

m is 0 or an integer in the range 1 to 3; and

n is an integer in the range1 to 3.

Another small molecule inhibitors of prenyltransferases are the quinolinone derivatives disclosed in PCT publication WO97/21701. Inhibitors suitable for use in the subject method may be selected from amongst these compounds, e.g., having a structure represented in the general formula:



wherein

X is O or S;

R_{351} is H, alkyl, aryl, $-(CH_2)_m-C(=O)-R_{359}$, $-(CH_2)_m-S(=O)-R_{359}$, $-(CH_2)_m-S(=O)_2-R_{359}$;

R_{352}, R_{353} and R_{366}, independently represent H, halo, hydroxyl amino, cyano, alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, or alkylsulfonylalkyl, or

R_{352} and R_{353}, when on adjacent positions, can be taken together to form a ring of 5 to 8 ring atoms;

R_{354} and R_{355} are each independently H, halo, hydroxyl amino, alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, $-(CH_2)_m-C(=O)-R_{359}$, $-(CH_2)_m-S(=O)-R_{359}$, or $-(CH_2)_m-S(=O)_2-R_{359}$;

R_{356} and R_{357} are each independently H, halo, cyano, alkyl, alkyloxy, aryl, aryloxy, alkylthio, alkylamino, or

R_{356} and R_{357}, when on adjacent positions, can be taken together to form a ring of 5 to 8 ring atoms

R_{358} is H, halo, hydroxyl amino, cyano, alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, $-O-R_{360}$, $-S-R_{360}$, $-N(R_{361})_2$;

R_{359}, independently for each occurrence, represents hydroxyl, alkyl, alkyloxy, amino or alkylamino;

R_{360}, independently for each occurrence, represents hydrogen, alkyl, alkylcarbonyl, aryl, arylalkyl, alyyloxycarbonylalkyl, -alkyl-OR_{361} or –alykyl-$N(R_{361})_2$;

R_{361}, independently for each occurrence, represents hydrogen, alkyl, aryl, or arylakyl;

R_{367} is hydrogen, halo, cyano, alkyl, alkyloxycarbonyl, or aryl;

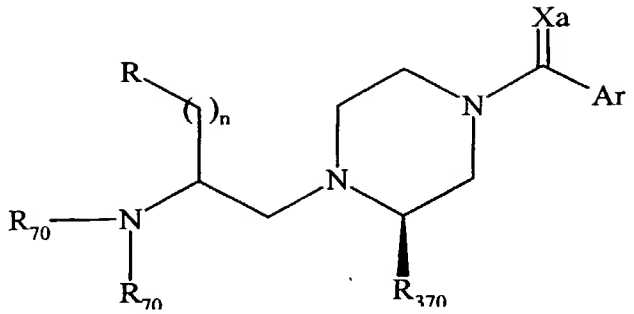
R_{368} is hydrogen, halo, alkyl, or alkyloxy;

R_{369} is hydrogen or alkyl; and

m is integer from 1 to 5.

Yet another class of non-peptide small molecule inhibitors of prenyltransferases are represented in the general formula:

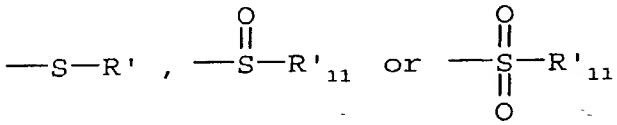
wherein



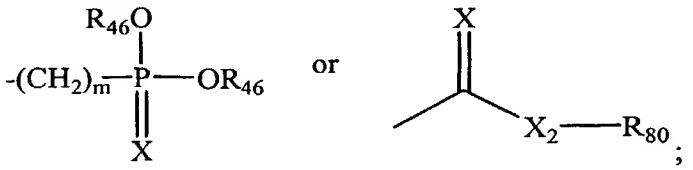
Ar represents an aryl group (e.g., substituted or unsubstituted);

X_a represents, independently for each occurrence, O, S or H_2

R represents



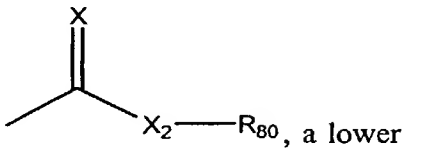
R' represents H, a lower alkyl, a lower alkenyl, an aryl,



R_7 represents an aryl, a cycloalkyl, a cycloalkenyl, or a heterocycle;

R'_{11} represents an alkyl, an alkenyl or $-(CH_2)_m-R_7$;

R_{46}, independently for each occurrence, represents hydrogen, a lower alkyl or an aryl;

R_{70}, independently for each occurrence, represents H,  , a lower alkyl, lower alkenyl, lower alkynyl, aryl, alkylaryl, cycloalkyl, alkoxyalkyl, alkylthioalkyl, hydroxyalkyl, aminoalkyl, carboxyalkyl, alkoxycarbonylalkyl, arylalkyl, alkylsulfonylalkyl, and an alpha-carbon sidechain of an amino acid residue or analog or other amino-protecting group, or a pharmaceutically acceptable salt or

R_{70} and R, or R_{70} and R_{70}, taken together form a 4 to 8 membered heterocycle;

R_{80} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, or $-(CH_2)_m-R_7$;

R_{370} represents an hydrogen, a lower alkyl, a lower alkenyl, a lower alkynyl, $-(CH_2)_m-$

O-lower alkyl, $-(CH_2)_m-O-R_7$, or $-(CH_2)_m-R_7$;

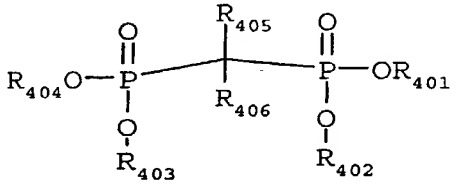
X represents, independently for each occurrence, O or S;

X_2 represents O or S ; and

m and n, independently for each occurrence, represent zero or an integer in the range of 1 to 4.

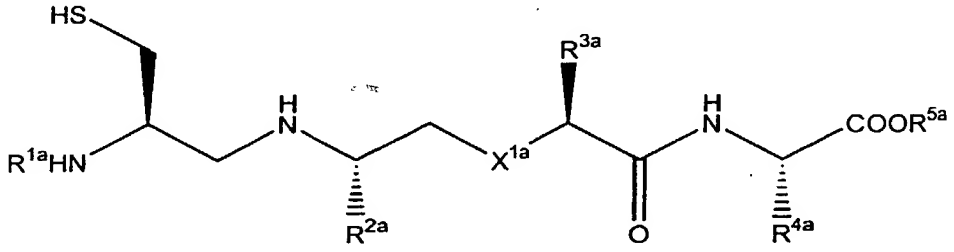
In preferred embodiments, R is –SR'; R' is H or lower alkyl, preferably H; Ar is C6-C12 aryl; R_{70} are each H; R_{370} is $-(CH_2)_2-O-CH_3$; X_a is O; n is 1.

Still another class of non-peptide small molecule inhibitors of prenyltransferases are the bisphosphonates disclosed in EP publication 537,008. Inhibitors suitable for use in the subject method may be selected from amongst these compounds, e.g., having a structure represented in the general formula:



wherein R_{401}, R_{402}, R_{403} and R_{404} each independently represent H, alkyl, aryl, alkylaryl, arylalkyl, ammonium, alkali metal or a prodrug ester.

Another group of prenyl transferase inhibitors is disclosed in the PCT publication WO 96/17623. The inhibitors of this publication are represented, in part, by the following general structure.



wherein

X^{1a} represents -O-, $-S(O)_m-$, $-N(R^{3a})-$, $-(CH_2)_2-$, or -CHCH-;

m is an integer of 0 to 2;

R^{1a} represents hydrogen, lower alkyl, aralkyl, acyl, lower alkylsulfonyl, aralkylsulfonyl, or arylsulfonyl;

R^{2a} represents lower alkyl;

R^{3a} represents lower alkyl, or aralkyl;

R^{4a} represents mercapto lower alkyl, lower alkylthio lower alkyl, lower alkylsulfinyl lower alkyl, lower alkylsulfonyl lower alkyl, or hydroxy lower alkyl;

R^{5a} represents hydrogen, or lower alkyl;

R^{4a} and R^{5a} may together form C_2 to C_4 alkylene.

The pharmaceutically acceptable salts of the subject prenyl transferase inhibitors include the conventional nontoxic salts or quaternary ammonium salts of the compounds, e.g., from non-toxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicyclic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, and the like.

The pharmaceutically acceptable salts of the present invention can be synthesized from the subject prenyl transferase inhibitor which contain a basic or acid moiety by conventional chemical methods. Generally, the salts are prepared by reacting the free base or acid with stoichiometric amounts or with an excess of the desired salt-forming inorganic or organic acid or base in a suitable solvent. The pharmaceutically acceptable salts of the acids of the subject prenyl transferase inhibitors are also readily prepared by conventional procedures such as treating an acid of the compound with an appropriate amount of a base such as an alkali or alkaline earth metal hydroxide (e.g. sodium, potassium, lithium, calcium or magnesium) or an organic base such as an amine, piperidine, pyrrolidine, benzylamine and the like, or a quaternary ammonium hydroxide such as tetramethylammonium hydroxide and the like.

Contemplated equivalents of the compounds described herein include compounds which otherwise correspond thereto, and which have the general properties thereof (e.g. the ability to inhibit a prenyl transferase), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound in inhibiting such enzymes.

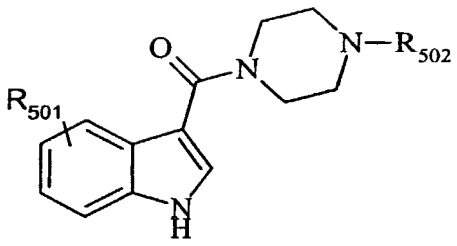
As is apparent from the present disclosure, other non-hydrolyzable peptide analogs can be generated which incorporate the basic structure of CAAM or CAAS. For illustrative purposes, peptide analogs of the present invention can be generated using, in addition to the

benzodiazepines described above, substituted gamma lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), β-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun*126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71), diaminoketones (Natarajan et al. (1984) *Biochem Biophys Res Commun* 124:141), and methyleneamino-modified (Roark et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

Other ras or GTPase inhibitors are described in, for example, European patent publications EP 520823 ; EP 523873 ; EP 528486 ; EP 537007 ; EP 537008 ; EP 618221 ; EP 675112 ; GB 2261374; Japanese patent publication JP 8239339; PCT publications WO 9410137; WO 9410138; WO 9617623; WO 9634113; WO 9705902; WO 9727852; WO 9731641; WO 9736584; WO 9736592; WO 9736876; WO 9736877; WO 9736881; WO 9736886; WO 9736888; WO 9736889; WO 9736891; WO 9736896; WO 9736897; WO 9736898; WO 9737678; WO 9738664; WO 9738697; and US Patents 5,322,855; 5,369,125; 5,420,245; 5,470,832; 5,498,627; 5,506,262; 5,567,729; 5,578,629; 5,686,472; 5,703,241; 5,770,731; 5,780,488; 5,780,492; and 5,783,593.

In other embodiments, the subject method utilizes an inhibitor of the kinase activity of raf, an MKK (Map kinase kinase) or a MAP kinase. The terms "mitogen activated protein kinase", "MAP kinase" and "MAPK" refer to protein kinases that are activated by dual phosphorylation on threonine and tyrosine and include among others: ERK1, ERK2, JNK-1, JNK-2, JNK-3, SAPK, p38, SMK1, HOG1, MPK1, FUS3/KSS1, and spk1. Exemplary inhibitors of this latter class are described in PCT publications WO98/15272, WO 98/20868 and WO 98/06715, and US Patents 5,849,733 and 5,525,625. For example, the method may utilize a broad spectrum inhibitor of MAP kinases, or specific inhibitors, such as the p38-specific inhibitor SB203580 or the MEK-specific inhibitor PD98059.

In one embodiment, the subject method provides an inhibitor represented by the general formula:

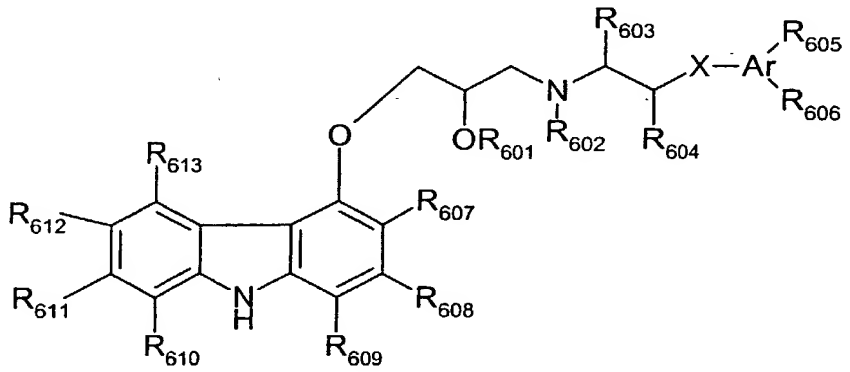


wherein

R_{501} is hydrogen, alkyl, aryl, heteroaryl, aryloxy, heteroaryloxy, nitro, amino, cyano, carboxy, carboxyalkoxy, carboamido, or halogen,

R_{502} is aryl, heteroaryl, arylalkyl, heteroarylalkyl, alkyl, cycloalkyl, or cycloalkyl.

In other embodiments, the inhibitor is a compound represented in the general formula



wherein,

R_{607}-R_{613} are, independently, hydrogen or -OH;

R_{601} is hydrogen, lower alkynoyl or aroyl (benzoyl or naphthoyl);

R_{602} is hydrogen, lower alkyl or arylalkyl;

R_{603} is hydrogen or lower alkyl;

R_{604} is hydrogen or lower alkyl, or when X is oxygen, R_{604} together with R_{605} can be $-CH_2-O-$;

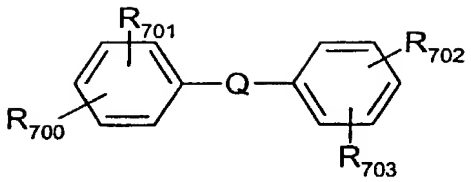
X is a single bond, $-CH_2-$, O or S;

Ar is selected from phenyl, naphthyl, indanyl and tetrahydronaphthyl;

R_{605} and R_{606} are individually selected from hydrogen, halide, -OH, lower alkyl, $-CONH_2$, lower alkoxyl, benzloy, alkylthio, lower alkylsulphinyl, lower alkylsulphonyl or

R_{605} and R_{606} taken together represent methylenedioxy.

In yet another embodiment, the subject inhibitor is represented in the general formula



wherein

R_{700} represents H, $NH_2(CNH)$-NH-N=CH-, or $NH_2(CNH)$-NH-N=$CHCH_3$-

R_{701}, R_{702} and R_{703}, independently, represents $NH_2(CNH)$-NH-N=CH-, or $NH_2(CNH)$-NH-N=$CHCH_3$-;

Q represents -NH(CO)NH-, -(C_6H_4)-, -(C_5NH_3)-, or -A-$(CH_2)_n$-A-;

A represents, independently for each occurrence, -NH(CO)-, -(CO)NH-, -NH(CO)NH-, -NH- or -O-; and

n is an integer from 2 to 10.

Preferably, when R_{700} represents $NH_2(CNH)$-NH-N=CH- or $NH_2(CNH)$-NH-N=$CHCH_3$-, it is meta or para to R_{701}, and R_{702} and R_{703} are meta or para to each other.

In still another embodiment, the inhibitor is a 2-(2-amino-3-methoxyphenyl)-4-oxo-4H-[1]benzopyran.

In addition to such small molecule inhibitors of ras pathway proteins, the present invention also contemplates the use of dominant negative mutants, antisense and other genetic suppressor elements which can inhibit or otherwise slow ras-dependent replicative senescence.

An *exemplary antisense construct for inhibiting ras expression is provided* Hamilton et al. (1998) Oncogene 16:1417. The use and design of exemplary antisense oligonucleotides of p38, JNK1, JNK2, ERK1 and ERK2 are taught by, e.g., Nagata et al. (1998) Blood 92:1859; and Yang et al. (1998) Hypertension 32:473.

An exemplary dominant negative ras inhibitor is the mutant Ha- ras (Leu-61; Ser-186). See, for example, Gaboli et al. (1995) J Gen Virol 76:751.

Examples of native proteins which, when ectopically expressed, can inhibit ras signaling including the Rap1 proteins. See, for example, Altschuler et al. (1998) PNAS 95:7475.

In yet other embodiments, the ras inhibitor can be a transcriptional repressor, or dominant negative mutant of a transcriptional activator, which inhibits expression of ras, or a downstream effector thereof, such as a MAP kinase or other positive regulator of ras-

dependent replicative senescence.

In other embodiments, the agents may inhibit other members of the small G protein superfamily, which consists of the Ras, Rho, Rab, Arf, Sar1, and Ran families.

As above, in preferred embodiments, the subject method use of such agents in a reversible manner, e.g., to return the ras pathway to its normal state after some period of time. Where the subject method makes use of a genetic construct for inhibition of a ras pathway, the construct is provided, as described above, with attributes which make the expression of a gene product inducible, transfection of the construct reversible, and/or the activity of the gene product inducible, etc.

(iv) Activation of Telomerase Activity

Considered as a whole, our analysis of lifespan limitations in most primary human cells indicates that both M0 and M1/M2 must be overcome to yield an immortal cell line. Accordingly, in preferred embodiments of the subject method, in addition to bypass of Rb/p16INK4a and/or inhibition of *ras*, the cells will also be treated with an agent that activates telomerase activity in cell.

In certain embodiments, the subject method relies on the ectopic expression of the telomerase catalytic subunit EST2, or a bioactive fragment thereof. In other embodiments, the subject method can be carried out by the ectopic expression of an activator of telomerase activity (collectively herein "telomerase activator") such as a *myc* gene product of a papillomavirus E6 protein. In preferred embodiments wherein the ectopic expression of the telomerase or telomerase activator involves a recombinant gene, expression of the gene in the host cell is inducible (or otherwise conditionally regulated) and/or the genetic construct including the gene can be readily removed from the host cell.

In still other embodiments, the subject method can be carried out by contacting the cell with an agent that inhibits degradation (ubiquitin-dependent or independent) of the EST2 protein or telomerase activator in order to increase the cellular half-life of the protein. For example, the method can utilize an agent which inhibits ubiquitination of to increase the cellular half-life of the protein. For example, the method can utilize an agent which inhibits ubiquitination of *myc* and thereby increases the cellular concentration of *myc*. In preferred embodiments, such agents are small, organic molecules, e.g., having molecular weights of less than 5000 amu (more preferably less than 1000 amu), and which are membrane permeant.

In still other embodiments, cellular proliferative capacity can be increased by contacting the cell with an agent, e.g. a small molecule, which relieves or otherwise inhibits a signal which antagonizes *myc*-induced activation of telomerase activity. For instance, agents

can be used which disrupt protein-protein interactions involved in inhibition of *myc* activity by, e.g., *mad-max* heterodimers.

The isolation of a gene the represents the human homolog, EST2, of the yeast and ciliate genes encoding the telomerase catalytic subunits has recently been reported. See Meyerson, et al. (1997) Cell 90:785; and Nakamura et al. (1997) Science 277:955.

The predicted 127 kDa protein shares extensive sequence similarity with the entire sequences of the Euplotes and yeast telomerase subunits and extends beyond the amino and carboxyl termini of these proteins. A BLAST search reveals that the probabilities of these similarities occurring by chance are 1.3×10^{-18} and 3×10^{-13}, respectively. By way of comparison, the probability of similarity between the yeast and Euplotes telomerases in a protein BLAST search is 6.9×10^{-6}. The human gene has been termed hEST2 (human EST2 homolog) to reflect its clear relationship with the yeast gene, the first of these genes to be described. EST2 was named because of the phenotype of Ever Shortening Telomerase catalytic subunit (Counter et al. (1997) *supra*; Lingner et al. (1997)).

Like the yeast and ciliate telomerase proteins, hEST2 is a member of the reverse transcriptase (RT) family of enzymes. Seven conserved sequence motifs, which define the polymerase domains of these enzymes, are shared among the otherwise highly divergent RT family (Poch et al. (1989) EMBO J 8:3867-3874; Xiong and Eickbush (1990) EMBO J 9:3353-3362). P123 and Est2p share six of these motifs with, most prominently, the a2-Sc enzyme, an RT that is encoded within the second intron of the yeast COX1 gene (Kennell et al. (1993) Cell 133-146). These six motifs, including the invariant aspartic acid residues known to be required for telomerase enzymatic function (Counter et al. (1997) *supra*; Lingner et al. *supra*), are found at the appropriate positions of the predicted sequence of hEST2. Thus, the proposed human telomerase catalytic subunit, like its yeast and ciliate counterparts, belongs to the RT superfamily of enzymes.

Exemplary human EST coding sequence and protein for use in the subject method is provided at GenBank accession AF018167, AF043739 and AF015950. Exemplary EST constructs are also described in PCT application WO98/14593 and Ulaner et al. (1998) Cancer Res 58:4168-72, Counter et L. (1998) Oncogene 161217-22, and Vaziri et al. (1998) Curr Biol 8: 279-82. In a preferred embodiment, the EST construct includes an EST coding sequence which hybridizes under stringent conditions to SEQ ID No: 1, or a coding sequence set forth in GenBank accession AF018167, AF043739 or AF015950. The EST coding sequence can encode an EST protein, or fragment thereof which retains a telomerase activity, which is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with a sequence of SEQ ID No. 2 or GenBank accession AF018167, AF043739 and AF015950, or identical with one of the enumerated sequences.

In other illustrative embodiments, telomerase activation can be caused by ectopic expression of a *myc* protein, e.g., c-*myc*. An exemplary human *myc* coding sequence is provided at the SWISS-PROT locus MYC_HUMAN, accession P01106. In a preferred embodiment, the *myc* construct includes an *myc* coding sequence which hybridizes under stringent conditions to a coding sequence set forth in SWISS-PROT locus MYC_HUMAN, accession P01106. The *myc* coding sequence can encode a *myc* protein, or fragment thereof which retains the ability to activate a telomerase activity, which is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with the protein sequence set forth in SWISS-PROT locus MYC_HUMAN, accession P01106, or identical thereto.

In yet other illustrative embodiments, telomerase activation is accomplished by expression of a papillomavirus E6 protein, preferably an E6 protein from a human papillomavirus (HPV), and more preferably an E6 protein from a high risk HPV (e.g., HPV-16 or -18). It may desirable to use an E6 protein which has been mutated so as to be incapable of effecting p53 degradation. In a preferred embodiment, the E6 construct includes an E6 coding sequence which hybridizes under stringent conditions to a coding sequence set forth in EMBL: locus A06324, accession A06324. The E6 coding sequence can encode an E6 protein, or fragment thereof which retains the ability to activate a telomerase activity, which is at least, for example, 60, 70 , 80, 85, 90 , 95 or 98 percent identical with the protein sequence set forth in EMBL: locus A06324, accession A06324, or identical thereto

In accordance with the subject method, expression constructs of the subject polypeptides may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *in vitro* or *in vivo* with a recombinant gene. As described above for Rb inactivators, approaches include insertion of the subject EST2 or telomerase activator gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out in vivo.

A preferred approach for introduction of nucleic acid encoding a telomerase activator into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which

have taken up viral vector nucleic acid. Preferred vectors include retrovirus vectors and adeno-associated virus vectors.

While the repair of telomers, e.g., by the activation of telomerase activity, can be enough for extending the replicative capacity of a cell, it can be a transforming event (e.g., to cause crisis and emergence of cancer cells), particularly where activation persists. Therefore, in one aspect, the present invention provides a method for increasing the proliferative capacity of cells, preferably normal cells, which method comprises delivering into the cell a gene construct which can *selectively* and *reversibly* activate telomerase activity in the cell.

Similar to the constructs described above for reversible expression of an Rb inactivator, in one embodiment, the coding sequence for the telomerase activator is provided as part of a vector which can be partially or completely excised from the host cell is an inducible manner.

The reversibility of telomerase activation can also be generated by use of an expression system which is inducible because of the presence of an inducible transcriptional regulatory sequence controlling the expression of the coding sequence of the EST or telomerase activator. Exemplary regulatable promoters include the tetracycline responsive promoters, such as described in, for example, Gossen et al. (1992) PNAS 89:5547-5551; and Pescini et al., (1994) Biochem. Biophys. Res. Comm. 202:1664-1667.

As described in further detail above, in other embodiments, the expression of the telomerase activator can be under the control of chimeric transcription factors which are dependent on small molecules "dimerizers" to assemble transcriptionally active complexes.

In other embodiments, the reversibility of telomerase activation can be accomplished by use of conditionally active (or conditionally inactivable) forms of EST or of the telomerase activators, such as temperature-sensitive mutants. Described supra.

In yet other embodiments, the multimerization technology referred to above can be used to generate small molecule inducible forms of EST or a telomerase activator. To illustrate, a first gene construct can be provided which encodes a fusion protein including a DNA binding domain (and optionally oligomerization domains) of myc and a ligand binding domain which binds to a small organic molecule, e.g., a domain which will bind to a dimerizing agent. A second gene construct is also provided, which construct encodes a fusion protein including an activation domain, e.g., a VP16 activation domain, and a ligand binding domain which will also bind the dimerizing agent when it is already bound to the first fusion protein. Expression of these two fusion proteins in a host cell, in the absence of the dimerizing agent, will not activate telomerase. Upon addition of the dimerizing agent, the fusion proteins associate, and activate transcription of genes which include myc responsive elements, which

causes activation of telomerase activity.

In yet another embodiment, ectopic expression of EST2 or other telomerase activator can be by way of a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous telomerase activator gene. For instance, the gene activation construct can replace the endogenous promoter of an EST2 gene with a heterologous promoter, e.g., one which causes constitutive expression of the EST2 gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In one embodiment, the gene activation construct includes recombinase sites such that the transcriptional regulatory sequences could be removed, or at least inactivated, upon treatment of the cells with a recombinase. As above, similar embodiments may employ unique restriction enzyme sites in place of the recombinase sites.

In yet another embodiment, membrane permeable drugs (e.g., preferably small organic molecules) can be identified which activate the expression of an endogenous telomerase activator gene, e.g., an EST2 or myc gene. In light of the availability of the genomic EST2 and myc genes, it will be possible to produce reporter constructs in which a reporter gene is operably linked to the transcriptional regulatory sequence of the gene. When transfected into cells which possess the appropriate intracellular machinery for activation of the reporter construct through the regulatory sequence, the resulting cells can be used in a cell-based approach for identifying such compounds.

In embodiments wherein the cells are treated in culture, RNA encoding EST2, *myc* or another telomerase activator can be introduced directly into the cell, e.g., from RNA generated by *in vitro* transcription. In preferred embodiments, the RNA is preferably a modified polynucleotide which is resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases.

In still another embodiment of the subject method, the telomerase activator polypeptide can be contacted with a cell under conditions wherein the protein is taken up by the cell, e.g., internalized, without the need for recombinant expression in the cell. For instance, in the application of the subject method to skin, mucosa and the like, a variety of techniques have been developed for the transcytotic delivery of ectopically added proteins. As described above for Rb inactivators, the telomerase activator can be provided for transmucosal or transdermal delivery. In other embodiments, the polypeptide is provided as a chimeric polypeptide which includes a heterologous peptide sequence ("internalizing peptide") which drives the translocation of an extracellular form of a therapeutic polypeptide sequence across a

cell membrane in order to facilitate intracellular localization of the therapeutic polypeptide.

In other embodiments, the subject method employs small, organic molecules, e.g., having a molecular weight of less than 5000 amu, more preferably less than 1000 amu, and even more preferably less than 500 amu. Moreover, such compounds are preferably membrane permeant, e.g., able to diffuse across the cell membrane into the host cell when added directly to culture cells or cells in whole blood.

In this regard, the art provides examples of assays for identifying agents which are capable of activating telomerase activity, e.g., see US Patents 5,837,453, 5,830,644, 5,804,380 and 5,686,245.

In yet another embodiment, to the extent it is relevant, the intracellular level of TRT or a telomerase activator (protein) can be upregulated by inhibiting its natural turnover rate. For example, inhibitors of ubiquitin-dependent or independent degradation of the protein can be used to cause ectopic expression of protein in the sense that the concentration of the protein in the cell can be artificially elevated. Assays for detecting inhibitors of ubiquitination, e.g., which can be readily adapted for detecting inhibitors of ubiquitination of *myc* or other telomerase activators, are described in the literature, as for example US Patents 5,744,343, 5,847,094, 5,847,076, 5,834,487, 5,817,494, 5,780,454 and 5,766,927. Likewise, to the extent that other post-translational modifications, such as phosphorylation, influence protein stability, the present invention contemplates the use of inhibitors of such modifications, including, as appropriate, kinase or phosphatase inhibitors.

In still other embodiments, cellular proliferative capacity can be increased by contacting the cell with an agent, e.g. a small molecule, which relieves or otherwise inhibits a signal which antagonizes *myc*-induced activation of telomerase activity. For instance, agents can be used which disrupt protein-protein interactions involved in inhibition of *myc* activity by, e.g., *mad-max* heterodimers.

(v) Use of antioxidants

In still another embodiment, the subject method also utilizes an antioxidant for increasing the proliferative capacity. In one embodiment, the method utilizes an enzymatic antioxidant systems, e.g., such as reactive-oxygen scavenger enzymes of the oxidoreductase classification (i.e. enzymes classified under the Enzyme Classification number E.C. 1 (Oxidoreductases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB)) include oxidoreductases within this group. Examples include oxidoreductases selected from those classified under the

Enzyme Classification (E.C.) numbers: Glycerol-3-phosphate dehydrogenase [NAD +] (1.1.1.8), Glycerol-3-phosphate dehydrogenase [NAD(P)< + >] (1.1.1.94), Glycerol-3-phosphate 1-dehydrogenase [NADP] (1.1.1.94), Glucose oxidase (1.1.3.4), Hexose oxidase (1.1.3.5), Catechol oxidase (1.1.3.14), Bilirubin oxidase (1.3.3.5), Alanine dehydrogenase (1.4.1.1), Glutamate dehydrogenase (1.4.1.2), Glutamate dehydrogenase [NAD(P)< + >] (1.4.1.3), Glutamate dehydrogenase [NADP< + >] (1.4.1.4), L-Amino acid dehydrogenase (1.4.1.5), Serine dehydrogenase (1.4.1.7), Valline dehydrogenase [NADP< + >] (1.4.1.8), Leucine dehydrogenase (1.4.1.9), Glycine dehydrogenase (1.4.1.10), L-Amino-acid oxidase (1.4.3.2.), D-Amino-acid oxidase(1.4.3.3), L-Glutamate oxidase (1.4.3.11), Protein-lysine 6-oxidase (1.4.3.13), L-lysine oxidase (1.4.3.14), L-Aspartate oxidase (1.4.3.16), D-amino-acid dehydrogenase (1.4.99.1), Protein disulfide reductase (1.6.4.4), Thioredoxin reductase (1.6.4.5), Protein disulfide reductase (glutathione) (1.8.4.2), Laccase (1.10.3.2), Catalase (1.11.1.6), Peroxidase (1.11.1.7), Lipoxygenase (1.13.11.12), Superoxide dismutase (1.15.1.1)

In a preferred embodiment, the enzymatic system utilizes superoxide dismutase, catalase and glutathione peroxidase. The enzyme can be added directly to the culture, or as appropriate, be recombinantly expressed by the cultured cells.

In other embodiments, the system utilizes an organic or inorganic small molecule antioxidant. Exemplary antioxidants include β-carotene, vitamins C and E, selenium and cysteine, glutathione, bioflavanoids, sodium bisulfite, N-acetyl cysteine (NAC, a cell permeable antioxidant), diethyldithiocarbamate, 4-methylthiobenzoic acid, ebselen, lipoic acid,cysteine, methionine, 2-mercaptoethanol and/or photosensitizing molecules, e.g., agents which neutralized H_2O_2 and other oxygen radicals. In yet other embodiments, the agent is dismutase activator or mimietic. Such agents include Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), a cell-permeable superoxide dismutase (SOD) mimetic.

(vi) Inactivation of p53 pathway

In another embodiment, the subject invention utilizes an agent which inhibits the tumor suppressing activity of p53 as part of a method for increasing the proliferative capacity.

For instance, the agent can be one inhibits the expression of p53, such as a small organic molecule which inhibits transcription of the p53 gene, or can be an antisense molecule which inhibits transcription and/or translation of the p53 gene (or the related p63 or p73 genes). In one embodiment, the agent is an antisense nucleic acid which hybridizes under

stringent conditions to the coding sequence of the human p53 gene shown in SE ID No. 10, of the complement thereof.

The agent may also be selected from amongst those agents which inhibit p53-mediated gene expression, e.g., by interfering with p53-p53 or p53-DNA interactions. In other embodiments, the agent can be one which promotes ubiquitination or ubiquitin-dependent degradation of p53.

In still other embodiments, the subject method utilizes a dominant negative p53 protein, e.g., which expressed from a recombinant construct transfected into the target cells, or is introduced into the cell as a protein therapeutic, e.g., utilizing the transcytosis peptides described above.

In other illustrative embodiments, the agent can be one which inhibits p19(ARF). The INK4a-ARF locus encodes two distinct tumor suppressors, p16INK4a and p19(ARF). Whereas p16INK4a restrains cell growth through preventing phosphorylation of the retinoblastoma protein, p19(ARF) acts by attenuating Mdm2-mediated degradation of p53, thereby stabilizing p53. Recent data indicate that Mdm2 shuttles between the nucleus and the cytoplasm and that nucleo-cytoplasmic shuttling of Mdm2 is essential for Mdm2's ability to promote p53 degradation. See, e.g., Tao et al. (1999) PNAS 96:6937. Therefore, Mdm2 must export p53 from the nucleus to the cytoplasm where it targets p53 for degradation. Coexpression of p19(ARF) blocks the nucleo-cytoplasmic shuttling of Mdm2, and thereby stabilizes p53 by inhibiting the nuclear export of Mdm2.

As above, the agent can be one which inhibits expression of p19(ARF), e.g., such as a p19(ARF) antisense constructs which hybridizes to SEQ ID No. 11 or the complement thereof.

In other embodiments, the subject method utilizes a dominant negative p53 protein, e.g., which expressed from a recombinant construct transfected into the target cells, or is introduced into the cell as a protein therapeutic, e.g., utilizing the transcytosis peptides described above.

In still other embodiments, the agent can be a molecule which interferes with p19-dependent stabilization of p53, e.g., by inhibiting p19-dependent nucleo-cytoplasmic shuttling of Mdm2. For that matter, the agent can be one which inhibits Mdm2-dependent stabilization of p53 by any mechanism.

(vii) Inactivation of NF-kB pathway

In yet another embodiment, the subject method makes use of inhibitors of NF-kB mediated gene activation.

Pharmacological and genetic inhibition of transcription factor NF-kB protected cells from hydrogen peroxide-elicited cell death. This detrimental effect of NF-kB mediating hydrogen peroxide-induced cell death presumably relies on the induced expression of death effector genes such as p53, which was NF-kB-dependently upregulated in the presence of H_2O_2. Thus, NF-kB is linked to p53-dependent replicative senescence by its ability to upregulate p53 and its apparent link to oxidation states of the cell.

Moreover, IkB-α, the cytosolic inhibit of NF-kB-dependent transcription activation, when overexpressed in a cell results in an enrichment of c-Myc in the nucleoli, although the total amount of c-Myc protein was unchanged. Thus, NF-kB is also implicated as a negative regulator of telomerase activation.

Accordingly, inhibitors of NF-kB activation are contemplated by the present invention to be useful for overcoming replicative senescence. For example, the subject method can be practiced with agents that potentiate IkB inhibition of NF-kB nuclear localization and transcription activity. For instance, the agents may be those which inhibit phosphorylation of IkB, inhibit ubiquitination of IkB or potentiate the interaction of NF-kB and IkB. The agent may also be one which inhibits nuclear localization of NF-kB, or which inhibits NF-kB transcriptional activation, e.g., by inhibiting NF-kB-DNA or NF-kB-protein interactions.

(viii) Reversible Inactivation of Apoptosis

In certain embodiments, the subject method will include the reversible inactivation of apoptotic pathways. A wide range of apoptosis inhibitors have been described in the art. These include small molecule inhibitors of ICE proteases, inhibitors of caspases, Inhibitors of phospholipase A2, and the like. US Patent 5,869,519, describes C-terminal modified (n-substituted)-2-indolyl dipeptides as inhibitors of the ICE/ced-3 family of cysteine proteases.

(ix) Conjoint Applications

Another aspect of the invention provides a conjoint therapy wherein one or more other therapeutic agents are administered with the Rb inactivator or ras inhibitor agent(s). Such conjoint treatment may be achieved by way of the simultaneous, sequential or separate dosing of the individual components of the treatment. For example, the telomerase-activating therapeutic agent can be administered conjointly with a growth factors and other mitogenic agents. Mitogenic agent, as used herein, refers to any compound or composition, including peptides, proteins, and glycoproteins, which is capable of stimulating proliferation of a target cell population. For example, the telomerase-activating therapeutic agent can be conjointly administered with a T-cell mitogenic agent such as lectins, e.g., concanavalin A or

phytohemagglutinin. Other exemplary mitogenic agents include insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and certain of the transforming growth factors (TGFs).

In one embodiment, the subject method includes co-administration of an agent that relieves "capping" inhibition of EST2 rescue. We have noticed that EST2 will neither extend telomere length nor lifespan in late-passage HMEC cells, and certain other cell lines such as fibroblasts. While not wishing to be bound by any particular theory, this inability to extend telomeres in such cells may be the result of reaction kinetics –e.g., telomere binding proteins such as TRF (TTAGGG repeat binding factor) become abundant relevant to the telomeric sequences. The increased loading of telomeres with such proteins inhibits elongation induced by ectopic EST2. Such relative overabundance of proteins to telomers may be the result of, for example, reduction in the number of telomeric sequences relative to a constant concentration of associated proteins, increased expression (or stability) of the associated proteins, or a combination thereof. To alleviate such kinetic inhibition of EST2 activity, the cells can be treated with an oligonucleotide which competes (e.g., as a decoy) with the telomeres for binding of the telomere binding proteins. See, for example, Wright et al. (1996) EMBO J 15: 1734. In other embodiments, a dominant negative mutant of a telomere binding protein can be introduced into the cell in order to inhibit the formation of inhibitory protein complexes with the telomeric sequences. See, for example, Bianchi et al. (1997) EMBO J 16:1785-94; Broccoli et al. (1997) Hum Mol Genet 6: 69-76; Smith et al. (1997) Trends Genet 13:21-26; Zhong et al, (1992) Mol. Cell. Biol. 12:4834-4843; Chong et al. (1995) Science 270:1663-166). In still other embodiments, the agent can be an inhibitor of expression of a telomere binding proteins, such as antisense or a small molecule inhibitor of transcription of the gene. In yet other embodiments, such agents, particularly small molecules, can be identified by their ability to directly inhibit the formation of telomeric complexes including telomere binding proteins.

(x) Exemplary Ex vivo therapy

The present method can be used to increase the proliferative capacity of cells *in vivo*, *in vitro* and as part of an ex vivo protocol. While the method of the invention is applicable to any normal cell type, the method is preferably practiced using normal cells that express a low level of telomerase activity. For purposes of the present invention, the term "normal" refers to cells other than tumor cells, cancer calls, or transformed cells. An exemplary cell is an embryonic stem cells, such as disclosed in Thomson et al. (1998) Science 282:1145 and Shamblott et al. (1998) PNAS 95:13726. Especially preferred cells for use in the present method include embryonic, fetal, neonatal, and adult stem cells of any organ, and adult pluripotent

hematopoietic stem cells.

In one embodiment, the cells are stem and/or progenitor cells. These include hematopoietic stem cells, e.g., which are derived from bone marrow, mobilized peripheral blood cells, or cord blood. In other embodiments, the cells are progenitor cells for pancreatic or hepatic tissue, or other tissue deriving from the primative gut. In still other embodiments, the stem is a neuronal stem cell, such as neural crest which can be used to form neurons or smooth muscle cells.

In other embodiments, the cells are not stem or progenitor cells, e.g., they are committed cells, such as pancreatic β cells, smooth muscle cells (or other myocytic cells), fibroblasts, lymphocytic cells, e.g., B or T cells, osteocytes or chondrocytes, to name but a few.

While the subject method can be used either *in vivo* or *in vitro*, the invention has particular application to the cultivation of cells *ex vivo*, and provides especially important benefits to therapeutic methods in which cells are cultured *ex vivo* and then reintroduced to a host. For example, the subject method can be used to extend the proliferative capacity of cells which are harvested, or otherwise isolated in culture, which are to be transplanted to a patient.

Such protocols can find use in bone marrow transplants wherein bone marrow, or isolated hematopoietic progenitor cells are treated according to the present invention, with the inactivation of Rb and/or ras, and activation of telomerase, being reverted to the wild-type phenotype before, or shortly after, transplantation.

The subject method can also be used to extend T cell life in HIV and Down's patients.

It also has application in protocols for the formation of artificial tissues such as prosthetic devices, e.g., deriving from stem or committed cells. Exemplary tissues include pancreatic, hepatic, neural, myocytic, cartilaginous and osseous tissue.

To illustrate, the subject method can be used to enhance the lifespan of a hematopoietic cells and hematopoietic stem/progenitor cells. The term "hematopoietic cells" herein refers to fully differentiated myeloid cells such as erythrocytes or red blood cells, megakaryocytes, monocytes, granulocytes, and eosinophils, as well as fully differentiated lymphoid cells such as B lymphocytes and T lymphocytes. Thus, a hematopoietic stem/progenitor cell includes the various hematopoietic precursor cells from which these differentiated cells develop, such as BFU-E (burst-forming units-erythroid), CFU-E (colony forming unit-erythroid), CFU-Meg (colony forming unit-megakaryocyte), CFU-GM (colony forming unit-granulocyte-monocyte), CFU-Eo (colony forming unit-eosinophil), and CFU-GEMM (colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte).

In another embodiment, the subject method can be use to extend the lifespan of a pancreatic cells and pancreatic stem/progenitor cells. The term "pancreatic progenitor cell" refers to a cell which can differentiate into a cell of pancreatic lineage, e.g. a cell which can produce a hormone or enzyme normally produced by a pancreatic cell. For instance, a pancreatic progenitor cell may be caused to differentiate, at least partially, into α, β, δ, or ϕ islet cell, or a cell of exocrine fate. The pancreatic progenitor cells of the invention can also be cultured prior to administration to a subject under conditions which promote cell proliferation and differentiation. These conditions include culturing the cells to allow proliferation and confluence *in vitro* at which time the cells can be made to form pseudo islet-like aggregates or clusters and secrete insulin, glucagon, and somatostatin.

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells- α, β, δ, and ϕ-have been identified in the islets. The α cells constitute about 20% of the cells found in pancreatic islets and produce the hormone glucagon. Glucagon acts on several tissues to make energy available in the intervals between feeding. In the liver, glucagon causes breakdown of glycogen and promotes gluconeogenesis from amino acid precursors. The δ cells produce somatostatin which acts in the pancreas to inhibit glucagon release and to decrease pancreatic exocrine secretion. The hormone pancreatic polypeptide is produced in the ϕ cells. This hormone inhibits pancreatic exocrine secretion of bicarbonate and enzymes, causes relaxation of the gallbladder, and decreases bile secretion. The most abundant cell in the islets, constituting 60-80% of the cells, is the β cell, which produces insulin. Insulin is known to cause the storage of excess nutrients arising during and shortly after feeding. The major target organs for insulin are the liver, muscle, and fat-organs specialized for storage of energy.

In an exemplary embodiment, the subject therapeutic agents can be used to extend the lifespan of implanted pancreatic tissue, e.g., implanted β-islet cells. Recently, tissue-engineering approaches to treatment have focused on transplanting pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Many methods for encapsulating cells are known in the art. For example, a source of β islet cells producing insulin is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the β islet cells (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng. 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the β islet cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotehnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials 12:50-55).

In any of the above-embodiments, the pancreatic cells can be treated by the subject method ex vivo, and/or treated by the subject method by subsequent delivery of an therapeutic to an animal in which the device is implanted. Such cells can be used for treatment of diabetes because they have the ability to differentiate into cells of pancreatic lineage, e.g., β islet cells. The pancreatic cells of the invention can be cultured *in vitro* under conditions which can further induce these cells to differentiate into mature pancreatic cells, or they can undergo differentiation in vivo once introduced into a subject.

Moreover, in addition to providing a source of implantable cells, either in the form of the progenitor cell population of the differentiated progeny thereof, the subject method can be used to extend the life of normal pancreatic cells used to produce cultures for the production and purification of secreted factors. For instance, cultured cells can be provided as a source of insulin. Likewise, exocrine cultures can be provided as a source for pancreatin.

In still another embodiment, the subject method can be used to extend the life span of hepatic cells and hepatic stem cells. The term "hepatic progenitor cell" as used herein refers to a cell which can differentiate in a cell of hepatic lineage, such a liver parenchymal cell, e.g., a hepatocyte. Hepatocytes are some of the most versatile cells in the body. Hepatocytes have both endocrine and exocrine functions, and synthesize and accumulate certain substance, detoxify others, and secrete others to perform enzymatic, transport, or hormonal activities. The main activities of liver cells include bile secretion, regulation of carbohydrate, lipid, and protein metabolism, storage of substances important in metabolism, degradation and secretion of hormones, and transformation and excretion of drugs and toxins. The subject method can be used to facilitate the long term culture of hepatic cells and hepatic progenitor cells either in vitro or subsequent to implantation.

In still another embodiment, the subject method can be used to enhance the life of "feeder" cell layers for cell co-cultures.

In another embodiment, the subject method can be used to enhance large-scale cloning, e.g., of non-human animals, by enhancing the presence of actively dividing fetal fibroblasts for nuclear transfer.

Prior research in nuclear transplantation has shown that the cell cycle stage of the donor cell affects the extent of development of the embryo after nuclear transfer. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated. This condensation phase has been shown to cause chromosomal defects in donor cells that are undergoing DNA synthesis. Donor cells in the G_1 phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development.

Our rationale in selecting an optimal donor cell for nuclear transplantation was that the cell should not have ceased dividing (which is the case in G_0) but be actively dividing, as an indication of a relatively undifferentiated state and for compatibility with the rapid cell divisions that occur during early embryo development. The cells should also be in G_1, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long G1 phase.

In certain embodiments, the subject method can be used to extend the lifespan of cells to such length that homologous recombination can be carried out in culture. Certain cells undergo replicative senescence too quickly for homologous recombination to be useful. In this regard, the subject method can be used as part of a protocol for generating transgenic animals, e.g., by knock-out or knock-in recombination, e.g., for animal husbandry.

The subject methods are also applicable to general cell culture techniques. For example, the method can be used to increase the replicative capacity of hybrids between immortal and mortal human cells, such as hybrids between human B-lymphocytes and myeloma cells, e.g., to increase the replicative capacity of antibody producing human hybridomas.

More generally, the subject method can be used to increase the replicative capacity of cells in culture which have been engineered to produce recombinant proteins. Indeed, the subject method can permit the use of "normal" cells as the recombinant cell, so that problems which may occur with the use of immortal cells (such as differences in post-translation modifications) can be avoided, particularly for producing secreted proteins.

In another aspect, the present invention provides pharmaceutical preparations and methods for controlling the proliferation of epithelially-derived tissue utilizing, as an active ingredient, one or more of (i) an Rb inactivator, (ii) a ras inhibitor, and/or (iii) a telomerase-activating therapeutic agent. The invention also relates to methods of controlling proliferation of epithelial-derived tissue by use of the pharmaceutical preparations of the invention. To illustrate, the Rb inactivators and ras inhibitors of the present invention may be used as part of regimens in the treatment of disorders of, or surgical or cosmetic repair of, such epithelial tissues as skin and skin organs; corneal, lens and other ocular tissue; mucosal membranes; and periodontal epithelium. The methods and compositions disclosed herein provide for the treatment or prevention of a variety of damaged epithelial and mucosal tissues. For instance, the subject method can be used to control wound healing processes, as for example may be desirable in connection with any surgery involving epithelial tissue, such as from dermatological or periodontal surgeries. Exemplary surgical repair for which use of Rb inactivators and ras inhibitors are candidate treatments include severe burn and skin regeneration, skin grafts, pressure sores, dermal ulcers, fissures, post surgery scar reduction,

and ulcerative colitis.

In another aspect of the present invention, Rb inactivators and ras inhibitors can be used to effect the growth of hair, as for example in the treatment of alopecia whereby hair growth is potentiated or otherwise extended.

Still another aspect of the present invention provides a method of extending the lifetime of epithelial tissue in tissue culture.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophegeal, epidermal, and hair follicle epithelial cells. Other exemplary epithlelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, which that characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g. tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the

epidermis which are deeply embedded in the dermis.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow; and "hair follicle epithelial cells" refers to epithelial cells which surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers which can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

"Dental tissue" refers to tissue in the mouth which is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.

"Internal epithelial tissue" refers to tissue inside the body which has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.

A "wound to eye tissue" refers to severe dry eye syndrome, corneal ulcers and abrasions and ophthalmic surgical wounds.

The subject method has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of contacting a cell, in vitro or in vivo, with an amount of an

telomerase-activating therapeutic agent sufficient to alter the life span of the treated epithelial tissue. For in vivo use, the mode of administration and dosage regimens will vary depending on the epithelial tissue(s) which is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues.

A method which "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment. "Promotion of wound healing" can also mean that the method causes the extends the proliferative and growth phase of, *inter alia,* keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g. the take rates of skin grafts,) when used together with the method of the present invention.

Complications are a constant risk with wounds that have not fully healed and remain open. Although most wounds heal quickly without treatment, some types of wounds resist healing. Wounds which cover large surface areas also remain open for extended periods of time. In one embodiment of the present invention, the subject method can be used to enhance and/or otherwise accelerate the healing of wounds involving epithelial tissues, such as resulting from surgery, burns, inflammation or irritation. The Rb inactivators and ras inhibitors of the present invention can also be applied prophylactically, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

Full and partial thickness burns are an example of a wound type which often covers large surface areas and therefore requires prolonged periods of time to heal. As a result, life-threatening complications such as infection and loss of bodily fluids often arise. In addition, healing in burns is often disorderly, resulting in scarring and disfigurement. In some cases wound contraction due to excessive collagen deposition results in reduced mobility of muscles in the vicinity of the wound. The compositions and method of the present invention can be used to enhance the healing of burns and to promote healing processes that result in more desirable cosmetic outcomes and less wound contraction and scarring.

Severe burns which cover large areas are often treated by skin autografts taken from undamaged areas of the patient's body. The subject method can also be used in conjunction with skin grafts to improve the grafts performance and life span in culture, as well as improve the "take" rates of the graft by accelerating growth of both the grafted skin and the patient's skin that is proximal to the graft.

Dermal ulcers are yet another example of wounds that are amenable to treatment by the subject method, e.g., to cause healing of the ulcer and/or to prevent the ulcer from becoming a

chronic wound. For example, one in seven individuals with diabetes develop dermal ulcers on their extremities, which are susceptible to infection. Individuals with infected diabetic ulcers often require hospitalization, intensive services, expensive antibiotics, and, in some cases, amputation. Dermal ulcers, such as those resulting from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) and arterial ulcers also resist healing. The prior art treatments are generally limited to keeping the wound protected, free of infection and, in some cases, to restore blood flow by vascular surgery. According to the present method, the afflicted area of skin can be treated by a therapy which includes an Rb inactivator and/or ras inhibitor which promotes epithelization of the wound, e.g., accelerates the rate of the healing of the skin ulcers.

In another exemplary embodiment, the subject method is provided for treating or preventing gastrointestinal diseases. Briefly, a wide variety of diseases are associated with disruption of the gastrointestinal epithelium or villi, including chemotherapy- and radiation-therapy-induced enteritis (i.e. gut toxicity) and mucositis, peptic ulcer disease, gastroenteritis and colitis, villus atrophic disorders, and the like. For example, chemotherapeutic agents and radiation therapy used in bone marrow transplantation and cancer therapy affect rapidly proliferating cells in both the hematopoietic tissues and small intestine, leading to severe and often dose-limiting toxicities. Damage to the small intestine mucosal barrier results in serious complications of bleeding and sepsis. The subject method can be used to promote proliferation of gastrointestinal epithelium and thereby increase the tolerated doses for radiation and chemotherapy agents. Effective treatment of gastrointestinal diseases may be determined by several criteria, including an enteritis score, other tests well known in the art.

With age, the epidermis thins and the skin appendages atrophy. Hair becomes sparse and sebaceous secretions decrease, with consequent susceptibility to dryness, chapping, and fissuring. The dermis diminishes with loss of elastic and collagen fibers. Moreover, keratinocyte proliferation (which is indicative of skin thickness and skin proliferative capacity) decreases with age. An increase, or prolonged rate of keratinocyte proliferation is believed to counteract skin aging, i.e., wrinkles, thickness, elasticity and repair. According to the present invention, Rb inactivators and ras inhibitors, and a telomerase-activating therapeutic agent can be used either therapeutically or cosmetically to counteract, at least for a time, the effects of aging on skin.

The subject method can also be used in treatment of a wound to eye tissue. Generally, damage to corneal tissue, whether by disease, surgery or injury, may affect epithelial and/or endothelial cells, depending on the nature of the wound. Corneal epithelial cells are the non-keratinized epithelial cells lining the external surface of the cornea and provide a protective barrier against the external environment. Corneal wound healing has been of concern to both

clinicians and researchers. Ophthalmologists are frequently confronted with corneal dystrophies and problematic injuries that result in persistent and recurrent epithelial erosion, often leading to permanent endothelial loss. The use of the subject method in these instances to promote epithelialization of the affected corneal tissue. To further illustrate, specific disorders typically associated with epithelial cell damage in the eye, and for which the subject method can provide beneficial treatment, include persistent corneal epithelial defects, recurrent erosions, neurotrophic corneal ulcers, keratoconjunctivitis sicca, microbial corneal ulcers, viral cornea ulcers, and the like. Moreover, superficial wounds such as scrapes, surface erosion, inflammation, etc. can cause lose of epithelial cells. According to the present invention, the corneal epithelium is contacted with an amount of one or more of (i) an Rb inactivator, (ii) a ras inhibitor, and (iii) a telomerase-activating therapeutic agent effective to enhance proliferation of the corneal epithelial cells to appropriately heal the wound.

The maintenance of tissues and organs *ex vivo* is also highly desirable. Tissue replacement therapy is well established in the treatment of human disease. For example, more than 40,000 corneal transplants were performed in the United States in 1996. Human epidermal cells can be grown in vitro and used to populate burn sites and chronic skin ulcers and other dermal wounds. The subject method can be used to enhance the life span of epithelial tissue *in vitro*, as well as to enhance the grafting of the cultured epithelial tissue to an animal host

The present method can be used for improving the "take rate" of a skin graft. Grafts of epidermal tissue can, if the take rate of the graft is to long, blister and shear, decreasing the likelihood that the autograft will "take", i.e. adhere to the wound and form a basement membrane with the underlying granulation tissue. Take rates can be increased by the subject method by enhancing the proliferation of the keratinocytes. The method of increasing take rates comprises contacting the skin autograft with an effective wound healing amount of one or more of (i) an Rb inactivator, (ii) a ras inhibitor, and (iii) a telomerase-activating therapeutic agent described in the method of promoting wound healing and in the method of promoting the growth and proliferation of keratinocytes, as described above.

Skin equivalents have many uses not only as a replacement for human or animal skin for skin grafting, but also as test skin for determining the effects of pharmaceutical substances and cosmetics on skin. A major difficulty in pharmacological, chemical and cosmetic testing is the difficulties in determining the efficacy and safety of the products on skin. One advantage of the skin equivalents of the invention is their use as an indicator of the effects produced by such substances through in vitro testing on test skin.

Thus, in one embodiment of the subject method can be used as part of a protocol for skin grafting of, e.g., denuded areas, granulating wounds and burns. The use of the subject

method can enhance such grafting techniques as split thickness autografts and epidermal autografts (cultured autogenic keratinocytes) and epidermal allografts (cultured allogenic keratinocytes). In the instance of the allograft, the use of the subject method to enhance the formation of skin equivalents in culture helps to provide/maintain a ready supply of such grafts (e.g., in tissue banks) so that the patients might be covered in a single procedure with a material which allows permanent healing to occur.

In this regard, the present invention also concerns composite living skin equivalents comprising an epidermal layer of cultured keratinocyte cells which have been expanded in the presence of one or more of (i) an Rb inactivator, (ii) a ras inhibitor, and (iii) a telomerase-activating therapeutic agent. The subject method can be used as part of a process for the preparation of composite living skin equivalents. In an illustrative embodiment, such a method comprises obtaining a skin sample, treating the skin sample enzymically to separate the epidermis from the dermis, treating the epidermis enzymically to release the keratinocyte cells, culturing, in the presence of a telomerase-activating therapeutic agent, the epidermal keratinocytes until confluence, in parallel, or separately, treating the dermis enzymatically to release the fibroblast cells, culturing the fibroblasts cells until sub-confluence, inoculating a porous, cross-linked collagen sponge membrane with the cultured fibroblast cells, incubating the inoculated collagen sponge on its surface to allow the growth of the fibroblast cells throughout the collagen sponge, and then inoculating it with cultured keratinocyte cells, and further incubating the composite skin equivalent complex in the presence of one or more of (i) an Rb inactivator, (ii) a ras inhibitor, and (iii) a telomerase-activating therapeutic agent to enhance the life span of the cells.

In other embodiments, skin sheets containing both epithelial and mesenchymal layers can be isolated in culture and expanded with culture media supplemented with one or more of (i) an Rb inactivator, (ii) a ras inhibitor, and (iii) a telomerase-activating therapeutic agent.

Any skin sample amenable to cell culture techniques can be used in accordance with the present invention. The skin samples may be autogenic or allogenic.

In another aspect of the invention, the subject method can be used in conjunction with various periodontal procedures in which control of epithelial cell proliferation in and around periodontal tissue is desired. In one embodiment, proliferative forms of the hedgehog and ptc therapeutics can be used to enhance reepithelialization around natural and prosthetic teeth, e.g., to promote formation of gum tissue.

In yet another aspect, the subject method can be used to help control guided tissue regeneration, such as when used in conjunction with bioresorptable materials. For example, incorporation of periodontal implants, such as prosthetic teeth, can be facilitated by the instant method. Reattachment of a tooth involves both formation of connective tissue fibers and re-

epithelization of the tooth pocket. The subject method treatment can be used to enhance tissue reattachment by controlling the mitotic capacity of basal epithelial cells in the wound healing process.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Telomere maintenance has been proposed as an essential prerequisite to human tumor development. The telomerase enzyme is itself a specific marker for tumor cells, but the genetic alterations that activate the enzyme during neoplastic transformation have remained a mystery. Amplification of the *myc* oncogene is prevalent in a broad spectrum of human tumors. Here, we show that *myc* induces telomerase both in normal human mammary epithelial cells (HMEC) and in normal human diploid fibroblasts. *Myc* increases expression of hEST2 (hEST/TP2), the catalytic subunit of telomerase. Since hEST2 limits enzyme activity in normal cells, *myc* may control telomerase solely by regulating hEST2 levels. Activation of telomerase through hEST2 is sufficient to increase average telomere length and extend lifespan in normal human mammary epithelial cells. Since *myc* can also extend the lifespan of these cells, activation of telomerase may be one mechanism by which *myc* contributes to tumor formation.

Telomerase activity is largely absent from somatic cells in vivo and from normal human cells in culture. As these cells proliferate, telomeric repeats are progressively lost due to the incomplete replication of chromosome ends during each division cycle. Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell toward the end of its replicative life-span. According to this model, erosion of chromosome ends triggers cellular senescence. Bypass of senescence through negation of tumor suppressor pathways (e.g. p53 and Rb/p16) allows continued proliferation and further loss of telomeric sequences. Indefinite proliferation in the absence of telomere maintenance would result in chromosomal destabilization due to complete loss of telomeres. Since this is probably incompatible with survival, cells with an indeterminate life span must adopt strategies for telomere conservation.

Stabilization of telomeric repeats has been proposed as a prerequisite for tumorigenesis. Circumstantial support for this notion comes from the observation that telomerase is activated in a high percentage of late-stage human tumors. The possibility that

telomere maintenance might be an essential component of the tumorigenic phenotype led us to survey known oncogenes for the ability to activate the telomerase enzyme.

Normal human mammary epithelial cells lack telomerase, whereas immortal HMEC-derivatives and breast tumor cell lines are almost universally telomerase-positive. Introduction into HMEC of HPV-16 E6 protein stimulates telomerase activity, suggesting that, in these cells, a single genetic event can potentiate the enzyme (Fig. 3). HMEC were therefore used for the oncogene survey. Ectopic expression of mdm-2 failed to induce telomerase, consistent with the observation that activation of telomerase by E6 is separable from the ability of E6 to promote the degradation of p53 (data not shown). Several other cellular and viral oncogenes, including E7, activated ras (V12) and all cdc25 isoforms, also failed to induce telomerase (Fig 3, data not shown). However, introduction of a c-*myc* expression cassette resulted in the appearance of telomerase activity in HMEC (Fig. 3). The enzyme was detectable within one passage after transduction of HMEC with a retrovirus that directs *myc* expression. Following drug selection of infected cells, the *myc*-expressing population contained levels of telomerase activity that approximated those seen in a random sample of breast carcinoma cell lines (Fig. 3; e. g. T47D).

Introduction of E6 into normal human diploid fibroblasts fails to activate telomerase (Fig. 4). Similar results were observed following transfer of either activated ras or a dominant-negative p53 allele (data not shown). However, telomerase was induced by transduction of either IMR-90 (Fig. 4) or WI-38 cells (not shown) with a retrovirus that directs *myc* expression. As with HMEC, activity was apparent immediately after infection, and following selection of the *myc*-expressing population, telomerase reached levels comparable to those seen in a telomerase-positive fibrosarcoma cell line, HT1080 (Fig. 4).

A recent report suggests that E6 can activate the *myc* promoter. This prompted us to ask whether E6 might regulate telomerase through an effect on *myc* expression. In HMEC, expression of E6 resulted in induction of *myc* to levels approaching those achieved upon transduction of HMEC with a retrovirus that directs *myc* expression (Fig. 5A). Surprisingly, E6-induced alterations in *myc* protein did not reflect changes in the abundance of *myc* mRNA (Fig. 5B), suggesting that control of *myc* expression by E6 must occur at the post-transcriptional level. In contrast, *myc* levels remained unaltered following expression of E6 in IMR-90 cells wherein E6 is incapable of activating telomerase (Fig. 5A). This result is consistent with a model in which E6 regulates telomerase in HMEC by altering the abundance of *myc*.

The presence of the mRNA encoding hEST2, the catalytic subunit of telomerase, strictly correlates with telomerase activity. The mRNA for hEST2 is undetectable in normal tissue and in normal cell lines, whereas hEST2 is present in immortal and tumor-derived cell

lines. Moreover, hEST2 expression and telomerase are concomitantly suppressed when cells are induced to differentiate. As expected, hEST2 mRNA was absent from normal HMEC. However, hEST2 could be detected in HMEC cells following transduction with a *myc* retrovirus (Fig. 6A). To determine whether increased expression of hEST2 was sufficient to account for activation of telomerase by *myc*, we infected HMEC and IMR-90 with a retrovirus that directs expression of hEST2. Delivery of hEST2 resulted in a clear induction of telomerase in both cell types (Fig. 6B). Considered together, our results indicate that *myc* regulates telomerase by controlling the expression of a limiting telomerase subunit. *Myc* is a transcription factor that can enhance the expression of responsive genes. Thus, *myc* could increase hEST2 expression by directly stimulating the hEST2 promoter. Alternatively, changes in hEST2 expression could arise as a secondary consequence of the ability of *myc* to regulate other genes.

Telomere length is regulated at two distinct levels. First, preservation of telomeric repeats requires either the telomerase enzyme or the activation of an alternative pathway for telomere maintenance. Second, telomere length can be controlled by telomere binding proteins. To determine whether activation of telomerase in HMEC cells is sufficient to stabilize telomere length, we followed telomeric restriction fragment (TRF) size as HMEC were passaged either in the presence or absence of telomerase activity. In normal HMEC, telomere length diminished slightly as cells underwent multiple rounds of division (Fig. 6C). Activation of telomerase by expression of hEST2 not only prevented telomere shrinkage but also increased average TRF length over that observed in early-passage cells (Fig. 6C).

Telomere length has been proposed as the counting mechanism that determines the replicative lifespan of a cell. Early-passage, normal HMEC which received either hEST2 or *myc* expression cassettes display extended lifespan as compared to vector-transduced cells (Fig. 6D). This supports the notion that telomere length is one of the criteria used by a cell to calculate its proliferative capacity.

Here we show that ectopic expression of *myc* can induce telomerase both in normal epithelial cells and in normal fibroblasts and can extend the replicative lifespan of HMEC. The *myc* oncogene is activated by gene amplification and possibly by mutation in a wide variety of different tumor types. Since *myc* can elevate telomerase to a level approximating that observed in tumor cell lines, increased *myc* activity could account for the presence of telomerase in many late-stage tumors. In this regard, a study of 100 neuroblastomas revealed that ~20% (16/100) had exceptionally high telomerase activity. Of these, 11 showed amplification of the N-*myc* locus. Thus, in this case, telomerase levels correlated well with *myc* activation. Although the *myc* oncogene may induce telomerase in significant proportion of tumors, the enzyme may also be regulated by other pathways.

Promotion of cell proliferation and oncogenic transformation by *myc* probably requires induction of a number of different target genes. As telomere maintenance may contribute to the long-term proliferative potential of tumor cells, telomerase activation may be an essential component of the ability of *myc* to facilitate tumor formation.

Methods

Retroviral plasmids. The following viral plasmids were used for transfection: pBabe-puro , MarXII-hygro, mouse c-*myc*/MarXII-hygro (gifts from Dr. P. Sun, CSHL), E6/pBabe-puro, cdc25A/MarXII-hygro. The full length hEST2 cDNA (a gift from Dr. R. Weinberg) was cloned into pBabe-puro vector at the EcoRI and SalI sites.

Cell culture and retroviral-mediated gene transfer. Human mammary epithelial cells (HMEC 184 spiral K) were obtained from Dr. M. Stampher. Normal human diploid fibroblasts (IMR90 and WI38) and human breast cancer cell lines (BT549, T47D and HBL100) were obtained from ATCC. HT1080 cells were a gift from G. Stark (Cleveland Clinic Foundation). The amphotropic packaging line, linX-A, was produced in our laboratory (L. Y. X, D. B. and G. H., unpublished). HMEC were cultured in complete MEGM (Clonetics). Fibroblasts and LinX-A cells were maintained in DMEM (GIBCO) plus 10% fetal bovine serum (FBS; Sigma). BT549, HBL100 and T47D were maintained as directed by the supplier. LinX-A cells were transfected by calcium-phosphate precipitation with a mixture containing 15 μg of retroviral plasmid and 15 μg of sonicated salmon sperm DNA. Transfected cells were incubated at 37°C for 24 hr and then shifted to 30°C for virus production. After 48 hr, the virus was collected, and the virus-containing medium was filtered to remove packaging cells (0.45 μm filter; Millipore). Target cells were infected with virus supernatants supplemented with 4 μg/ml polybrene (Sigma) by centrifuging for 1 hr at 1000 g and then incubating at 30°C overnight. The infected cells were selected 48 hours after infection using appropriate drugs (hygro*myc*in, G418 or puro*myc*in).

TRAP assays. Briefly, extracts were prepared in lysis buffer (10 mM Tris [pH 7.5], 1 mM $MgCl_2$, 1 mM EGTA, 10% Glycerol), and cleared by centrifugation for 30 min at 50,000xg. Lysate corresponding to from 10 to 10^4 cells was used in the assay. Telomeric repeats were synthesized onto an oligonucleotide, TS (5' AATCCGTCGAGCAGAGTT3'), in an extension reaction that proceeded at 30°C for 1 hr. Extension products were amplified by polymerase chain reaction (PCR) in the presence of ^{32}P-dATP using TS in combination with a downstream anchor primer (5' GCGCGGCTAACCCTAACCCTAACC 3'). Five microliters of each reaction was analyzed on a 6% acrylamide / 8 M urea gel.

Northern blotting. Total RNA was isolated from subconfluent cultures using Trizol

reagent (GIBCO BRL). Ten micrograms of total RNA was resolved by electrophoresis and transferred to Hybond-N+ membranes according to the manufacturer's instructions. hEST2 was visualized following hybridization with a labeled Stu I fragment of hEST2.

Western blotting. Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were incubated either with a c-*myc* rabbit polyclonal antibody (N-262; Santa Crutz) or with a TFIIB rabbit polyclonal antibody (a gift from Dr. B. Tansey). Immune complexes were visualize by secondary incubation with ^{125}I-protein A (ICN).

All of the above-cited references, publications and pending applications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

We Claim:

1. A method for increasing the proliferative capacity of cells, comprising contacting the cell with a first agent which reversibly activates telomerase activity in the cell, and second agent which reversibly inactivates one or both of an Rb/INK4 pathway or a p53 pathway.

2. A method for increasing the proliferative capacity of cells, comprising contacting the cell with a first agent which reversibly activates telomerase activity in the cell, and a second agent which reversibly inactivates a ras pathway.

3. A method for increasing the proliferative capacity of cells, comprising contacting the cell with an agent which decreases Rb-dependent cellular senescence.

4. A method for increasing the proliferative capacity of cells, comprising contacting the cell with an agent which decreases ras-dependent cellular senescence.

5. The method of claim 1, wherein the second agent is a polypeptide selected from the group consisting of:

MDM2, or a fragment thereof;

a dominant negative cdk4 or cdk6 mutants, e.g., which has lost the ability to bind and/or be inhibited by an INK4 protein, especially p16INK4a;

a dominant negative Rb mutant, e.g., as C pocket fragment;

a papillomavirus E7 protein, or other viral oncoprotein which bypasses Rb and/or p53, or fragment thereof;

a cyclin, preferably a cyclin active in G1 phase, such as cyclin D1 or cyclin E; and

a transcriptional repressor, or dominant negative mutant of a transcriptional activator, which inhibits expression of Rb, an INK4 protein or other positive regulator of Rb antiproliferative activity, such as the Bmi-1 gene product; and

6. The method of claim 5, wherein the polypeptide is provided as a conditionally active form to provide reversible inactivation of the Rb/p16 pathway.

7. The method of claim 1, wherein the second agent is an antisense molecule which inhibits p16 or Rb expression.

8. The method of claim 1, wherein the second agent is a small molecule inhibitors of Rb or p16 function, such as a small organic molecule that inhibits dephosphorylation of Rb, or at least formation of the hypophosphorylated for Rb (p115/hypo).

9. The method of claim 1, further comprising contacting the cell with an agent that inhibits ras-dependent replicative senescence.

10. The method of claim 9, wherein the agent is an inhibitor of a ras/Raf/MKK/MAP kinase pathway.

11. The method of claim 9, wherein the agent is an inhibitor of prenylation of ras.

12. The method of claim 9, wherein the agent is a dominant negative ras mutants, an

antisense inhibitor of ras expression or other genetic suppressor elements of ras, a Rap1 protein or fragment thereof.

13. The method of claim 1, wherein the first agent is selected from the group consisting of (i) an expression construct encoding an EST2 polypeptide or other telomerase activator protein, (ii) an agent which increases or activates expression of an endogenous EST2 gene, (iii) a telomerase activator polypeptide formulated for transcellular uptake, (iv) an agent which inhibits inactivation of endogenous an EST2 protein or *myc* protein, and (v) an agent which derepresses *myc*.

14. The method of claim 13, wherein the EST2 polypeptide is identical or homologous to SEQ ID No. 2, or wherein the EST2 polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

15. The method of claim 13, wherein the first agent is an RNA molecule encoding the telomerase activator.

16. The method of claim 13, wherein the first agent inhibits inactivation of an endogenous EST2 protein or *myc* protein by inhibiting post-translational modification of the protein and/or inhibiting proteolytic degradation of the protein.

17. The method of claim 16, wherein the agent inhibits ubiquitin-mediated degradation of *myc*.

18. The method of claim 13, wherein the agent depresses mad-dependent antagonism of *myc*.

19. The method of claim 5 or 14, wherein polypeptide is encoded by an expression construct delivered to the cell, the expression construct is a vector comprising

(i) one or more transposition elements for integration of the vector into chromosomal DNA of a eukaryotic host cell;

(ii) a coding sequence of for the polypeptide; and

(ii) excision elements for inactivating expression of the coding sequence upon contact with an excision agent.

20. The method of claim 19, wherein vector is a retroviral or lentiviral vector.

21. The method of claim 19 or 20, wherein the excision elements are recombinase recognition sites.

22. The method of claim 21, wherein the recombinase recognition sites are present in the transposition elements such that, upon contacting the cell with the excision agent, all or substantially all of the vector is excised from the chromosome of the cell.

23. The method of claim 1-4, wherein the cell is a stem cell or progenitor cells.

24. The method of any of claims 1-4, wherein the cell is contacted with the agent in a culture or in *ex vivo* explant.

25. The method of any of claims 1-4, wherein the agents are small organic molecules.

26. The method of any of claims 1-4, wherein the cell is a stem cell or progenitor cells.

27. The method of claim 26, wherein the cell is selected from the group consisting of

neuronal, hematopoietic, pancreatic, and hepatic stem and progenitor cells.

28. The method of any of claims 1-4, wherein the cell is an epithelial cell.

29. The method of any of claims 1-4, wherein the cell is a mesenchymal cell.

30. The method of any of claims 1-4, wherein the cell is a chondrocyte or osteocyte.

31. The method of any of claims 1-30, wherein the cell is contacted with the agent in a culture or in *ex vivo* explant.

32. The method of any of claims 1-30, wherein the cell is contacted with the agent *in vivo*.

33. The method of claim 32, wherein the agent is administered to a mammal.

34. The method of claim 33, wherein the mammal is a human.

35. The method of claim 33, wherein the agent is administered as a pharmaceutical preparation.

36. The method of claim 33, wherein the agent is administered as a cosmetic preparation.

37. A medicament formulated for increasing the proliferative capacity of cells, comprising an agent which reversibly activates telomerase activity in the cell, and an agent which reversibly inactivates one or both of an Rb/INK4 pathway or a p53 pathway.

38. A method for formulating a medicament for increasing the proliferative capacity of cells, comprising admixing an agent which reversibly activates telomerase activity in the cell, and an agent which reversibly inactivates one or both of an Rb/INK4 pathway or a p53 pathway.

39. A kit for increasing the proliferative capacity of cells, comprising a first agent which reversibly activates telomerase activity in the cell, and a second agent which reversibly inactivates one or both of an Rb/INK4 pathway or a p53 pathway, and optionally, directions for administering the first and second agents to a patient.

40. A cosmetic preparation comprising, as active components formulated in a pharmaceutically acceptable excipient for topical application, a first agent which reversibly activates telomerase activity in the cell, and a second agent which reversibly inactivates one or both of an Rb/INK4 pathway or a p53 pathway, in amounts suitable to promote proliferation of cells of a dermal layer when applied topically.

41. A kit for conjoint administration comprising, (a) the preparation of claim 37, the kit of claim 39 or the cosmetic preparation of claim 40, and (b) a trophic factor.

42. A kit for conjoint administration comprising, (a) the preparation of claim 37, the kit of claim 39 or the cosmetic preparation of claim 40, and (b) a tropic factor.

43. A kit for conjoint administration comprising, (a) the preparation of claim 37, the kit of claim 39 or the cosmetic preparation of claim 40, and (b) a mitogenic agent.

44. The kit of claim 43, wherein the mitogenic agent is a lectins, insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or a transforming growth factor (TGF).

45. The method of claim 1, wherein the first agent is co-administered with a second agent that relieves capping inhibition of EST2 rescue.

46. A method for *ex vivo* therapy comprising

(i) isolating, in cell culture, a population of cells which are to be transplanted to a patient;

(ii) contacting the cells with the preparation of claim 37, the kit of claim 39 or the cosmetic preparation of claim 40, in an amount sufficient to increase the number of mitotic divisions the cells can undergo in culture; and

(iii) transplanting the cells into the patient.

47. The method of claim 46, wherein the first and/or second agents are removed from the cells or inactivated before transplanting the cells into the patient.

48. A method for cloning a mammal, comprising

(i) obtaining desired differentiated mammalian cells to be used as a source of donor nuclei, and maintaining the donor cells in culture under conditions wherein telomerase catalytic activity of the donor cells is reversible activated and Rb/p16 pathways are reversibly inactivated so as to prevent replicative senescence;

(ii) transferring the desired differentiated cell or cell nucleus into an enucleated oocyte, under conditions suitable for the formation of NT units;

(iii) activating the resultant NT units;

(iv) culturing said activated nuclear transfer unit until greater than the 2-cell developmental stage; and

(vii) transferring said cultured NT unit to a host mammal such that the NT unit develops into a fetus.

Abstract

The present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In general, the subject method relies on the activation of a telomerase activity and inhibition of one or both of an Rb/p16 pathway or a p53 pathway.

```
1              MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGNRLVQRGDPAAFRAL  hEST2

51   VAQCLVCVPWDARPPPAAPSFRQVSCLKELVARVLQRLCERGAKNVLAFGFALLDGARGG  hEST2
1    MEVDVDNQADNHGIHSALKTCEEIKEAKTLYS-NIQKVI-RCRNQSQSHYKDLEDIKIFA  p123
1    --------------------------MKILFE-FIQDKLDIDLQTNSTYKENLKCGHFNG  Est2p

111  PPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRR-VGDDVLVHLLARCALFVLVAPSCAYQ  hEST2
59   QTNIVATP-RDYNEEDFKVIARKEVFSTGLMIE-LIDKCLVELLSS----SDVSDRQKLQ  p123
34   LDEILTT----CFALPNSRKIALPCLPGDLSHKAVIDHCIIYLL---------TGELYNN  Est2p

179  VCGPPLYQLGAATQARP-PPHASGPRRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSA  hEST2
113  CFG---FQLKGNQLAKTHLLTALSTQKQYFFQDEWN-QVR---------AMIGNELFRHL  p123
81   VLT------FGYKIAR---------------NEDVNNSL-------------------F  Est2p

229  SRSLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGA  hEST2
160  YTKYLIFQRTSEGTLVQ----FCGNNVFDHLKVNDKFDKK--------QKGGAADMNEPR  p123
100  CHSANVNVTLLKGAAWKMFHSLVGTYAFVDLLINYTVIQFN---GQFFTQIVGNRCNEPH  Est2p

289  LSGTRHSHPSVGRQHHAGPPSTSRPPRPNDTPCPPVYAETKHFLYSSGDK-EQLRPSFLL  hEST2
208  CCSTCKYNVKNEKDHF---LNNINVPN-NNN----MKSRTRIFYCTHFNRNNQFFKKHEF  p123
157  LPPKWVQRSSSSSATAA-QIKWLTEP--V---------TNKQFLHKLNINSSSFFPYSKI  Est2p

348  SSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPYGY  hEST2
260  VSNKNNISAMDR-AQTIF--TNIFRFNRIRK--KLKDKVIEKIAYMLEKVKDF---NFNY  p123
205  LPSSSSIKKLTDLREAIF-PTNLVKIPQ-----RLKVRINLTLQKLLKRHKRLN---YVS  ESt2p
```

Fig. 1A

```
408  LLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRRLVQLLRQHSSPWQVYG-FVR  hEST2
312  YLTKSCPL-----PENNRERKQKIENLINKTREEKS---KYYEELFSYTTDNKCVTQFIN  p123
256  ILNSICP------PLEGTVLD--------------------LSHLSRQSPKERVL-KFII  Est2p

467  ACLRRLVPPGLWGSRHNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRGCAWLRRSPGV  hEST2
364  EFFYNILPKDFLTGR-NRKNFQKKVKKYVELNKHELIHKNLLLEKINTREISWMQVETSA  p123
289  VILQKLLPQEMFGSKKNKGKIIKNLNLLLSLPLNGYLPFDSLLKKLRLKDFRWLFI--SD  Est2p

527  GCVPAAEHRLREEILAKFLHWLMSVYVVELLRSFFYVTETTFQKNRLFFYRKSVWSKLQS  hEST2
423  KHFYYFDHE-NIYVLWKLLRWIFEDLVVSLIRCFFYVTEQQKSYSKTYYYRKNIWDVIMK  p123
347  IWFTKHNFENLNQLAICFISWLFROLIPKIIQTFFYCTEISSTV-TIVYFRHDTWNKLIT  Est2p

                                    motif 1          motif 2
587  IGIRQHLKRVQLRELSEAEVRQHREAR--PALLTSRLRFIPK--PDGLRPI-VNMDY-VV  hEST2
482  MSI-ADLKKETLAEVQEKEVEEWKKS---LGFAPGKLRLIPK--KTTFRPI-MTFN----  p123
486  PFI-VEYFKTYLV---ENNVCRNHNSYTLSNFNHSKMRIIPKKSNNEFRIIAIPCRGADE  Est2p

641  GARTFRREKRAERLTSRVKALFSVLNYERARRPGL---LGASVLGLDDIHRAWRTFVLRV  hEST2
531  -KKIVNSDRKTTKLTTNTKLLNSHLMLKTLKNRMFKDPFGFAVFNYDDVMKKYEEFVCKW  p123
462  EEFTIYKENHKNAIOPTOKIL-EYLRNKRPTSFT--KIYSPTQIA-DRIKEFKQRLLKKF  ESt2p

                  motif 3
698  RAQDPPPELYFVKVDVTGAYDTIPQDRLTEVI--------------ASIIKPQNTYCVRR  hEST2
590  K-QVGQPKLFFATMDIEKCYDSVNREKLSTFLKTTKLLSSDFWIMTAQILKRKNNIVIDS  p123
518  --NNVLPELYFMKFDVKSCYDSIPRMECMRILK--------------DALKNENGFFVRS  Est2p
```

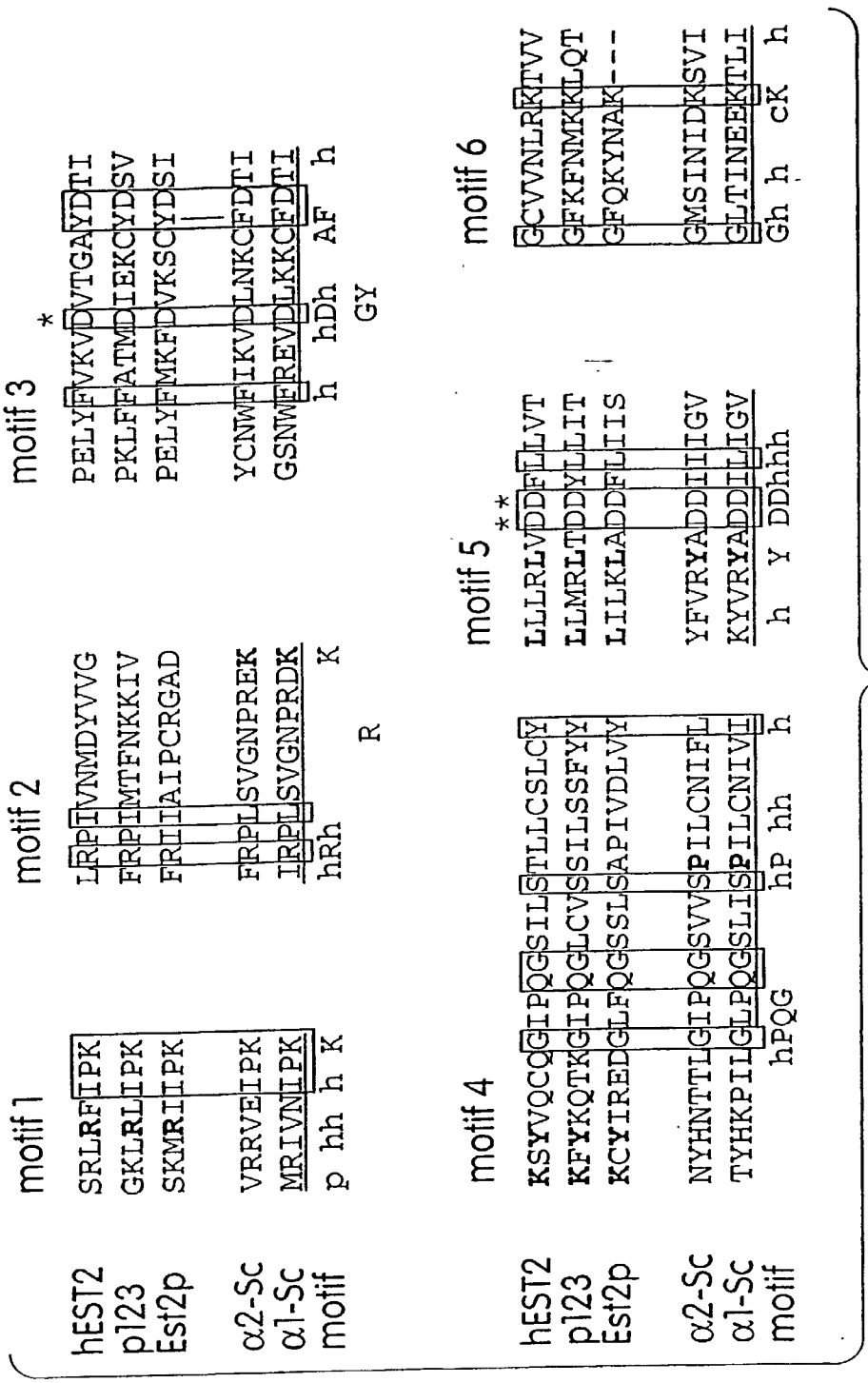
Fig. 1B

```
744  YAVVQKAAHGHVRKAFKSHVSTLTDLQ-PYMRQFVAHLQETSPLRDAVVIEQSSSLNEAS  hEST2
649  KNFRKKEMKDYFRQKFQK--IALEGGQYPTLFSVLENEQNDLNAKKTLIVEAKQRNYFKK  p123
562  QYFFNTNT------------GVL------KLFNVVNASRVPKPYE--LYIDNVRTVHLSN  Est2p

                              motif 4
803  SGLFDVFLRFMCHHAVRIRGKSYVQCQGIPQGSILSTLLCSLCYGDMENKLFAGIRRDG-  hEST2
707  DNLLQPVINICQYNYINFNGKFYKQTKGIPQGLCVSSILSSFYYATLEESSLGFLRDESM  p123
602  QDVINVVEMEIFKTALWVEDKCYIREDGLFQGSSLSAPIVDLVYDDLLE-FYSEFKASP-  Est2p

                motif 5                                  motif 6
862  --------LLLRLVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNLRKTVVNFPVEDEA  hEST2
767  NPENPNVNLLMRLTDDYLLITTQENNAVLFIEKLINVSRENGFKFNMKKLQTSFPLSPSK  p123
660  ----SQDTLILKLADDFLIISTDQQQ-VINIKKLAMG----GFQKYNAK-----ANRDKI  Est2p

914  LGGTAFVQMPAHGL----FPWCGLLLDTRTLEVQSDYSSYARTSIRASLTFN-RGFKAGR  hEST2
827  FAKYGMDSVEEQNIVQDYCDWIGISIDMKTLALMPNI-NLRIEGILCTLNLNMQTKKASM  p123
706  LAVSSQSDDDT------VIQFCAMHIFVKELEV------WKHSSTMNNFHIR---SKSSK  Est2p

969  NMRRKLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILLLQAYRFHACVLQLPFHQQVNKN  hEST2
886  WLKKKLKSFLMNNITHYF-RKTITTEDFANKTLNKLFISGGYKYMQCAKE--YKDHFKKN  p123
751  GIFRSLIALFNTRI-------SYKTIDTNLNSTNTVLMQIDHVVKNISE---CYKSAFKD  Est2p

1029 PTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKL-TRHRV  hEST2
943  LAMSSMIDLEVSKIIYSVTRAFFKYLVCNIKDTIFGEEHYPDFFLSTLKHFIEIFSTKKY  p123
801  LSINVTQNMQFHSFLQRIIEMTVSGCPITK---CDPLIEYEVRFTILNGFLESL-SSNTS  Est2p
```

Fig. 1C

hEST2	1088	TYVPLLGSLRTAQTQL-SRKLPGTTLTALEAAANPALPSDFKTILD
p123	1003	IFNRVCMILKAKEAKLKSDQC--QSLIQYDA
Est2p	857	KFKDNIILLRKEIQHLQAYIYIYIHIVN

Fig. 1D


motif 1
motif 2
motif 3
motif 4
motif 5
motif 6
hEST2
p123
Est2p
α2-Sc
α1-Sc
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GKLRLIPK
SKMRIIPK
VRRVEIPK
MRIVNIPK
p hh h K
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h hDh AF h
GY
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KCYIREDGLFQGSSLSAPIVDLVY
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TYHKPILGLPQGSLISPILCNIVI
hPQG hP hh h
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LLMRLTDDYLLIT
LILKLADDFLIIS
YFVRYADDIIIGV
KYVRYADDILIGV
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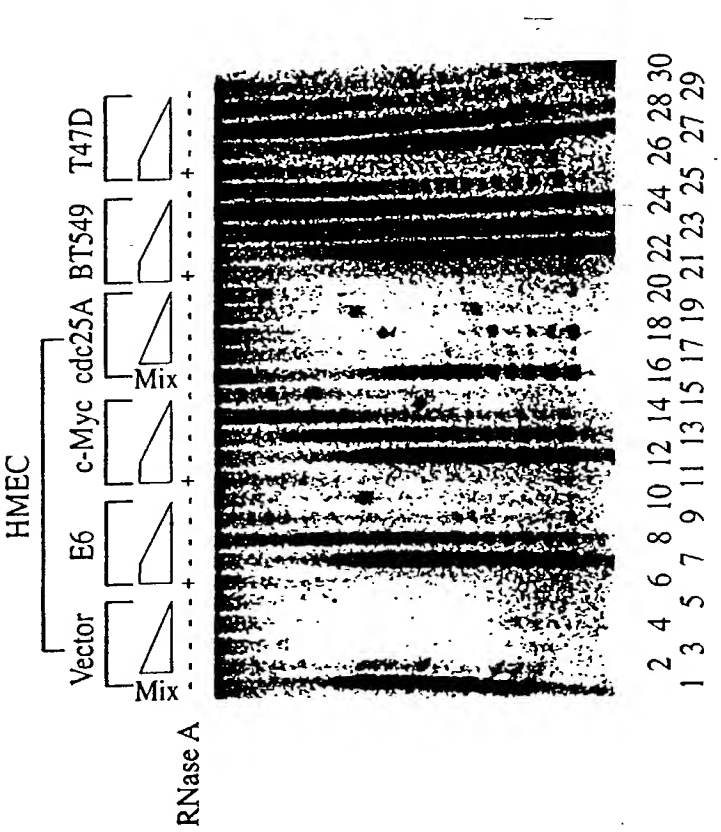
GLTINEEKTLI
Gh h cK h

Fig. 2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Vector
E6
c-Myc
cdc25A
BT549
T47D
Mix
Mix
RNase A
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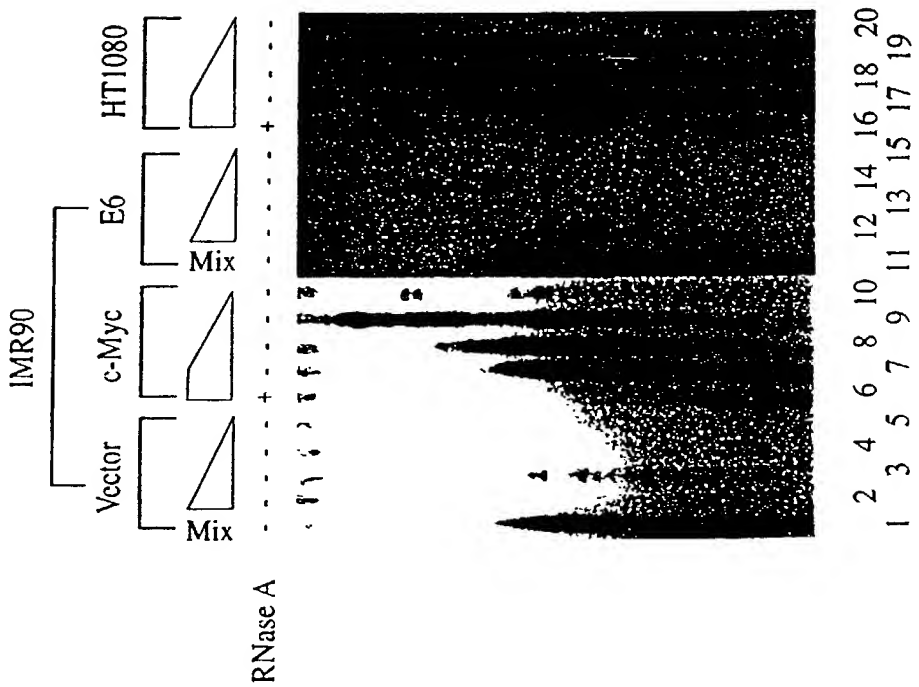
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Fig. 3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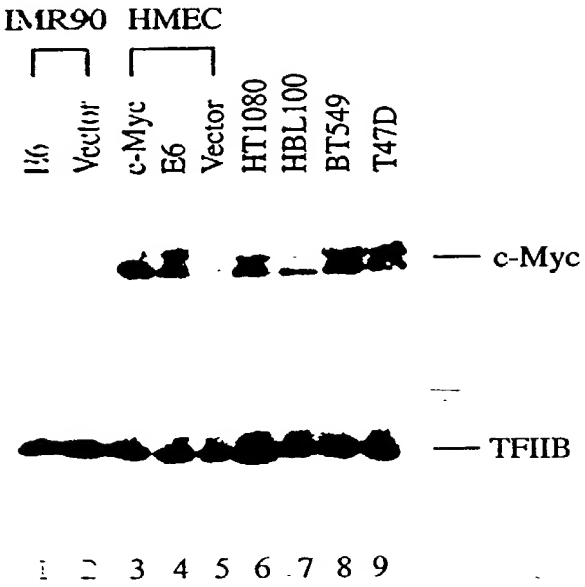
HT1080
Mix
Mix
RNase A
- - - - - + - - - - - - - - - - + - - - - -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. 4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HMEC
E6
Vector
c-Myc
E6
Vector
HT1080
HBL100
BT549
T47D
c-Myc
TFIIB
1 2 3 4 5 6 7 8 9

Fig. 5A

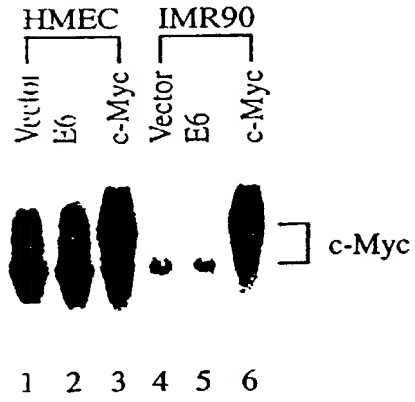
HMEC
IMR90
Vector
E6
c-Myc
Vector
E6
c-Myc
c-Myc
1 2 3 4 5 6

Fig. 5B

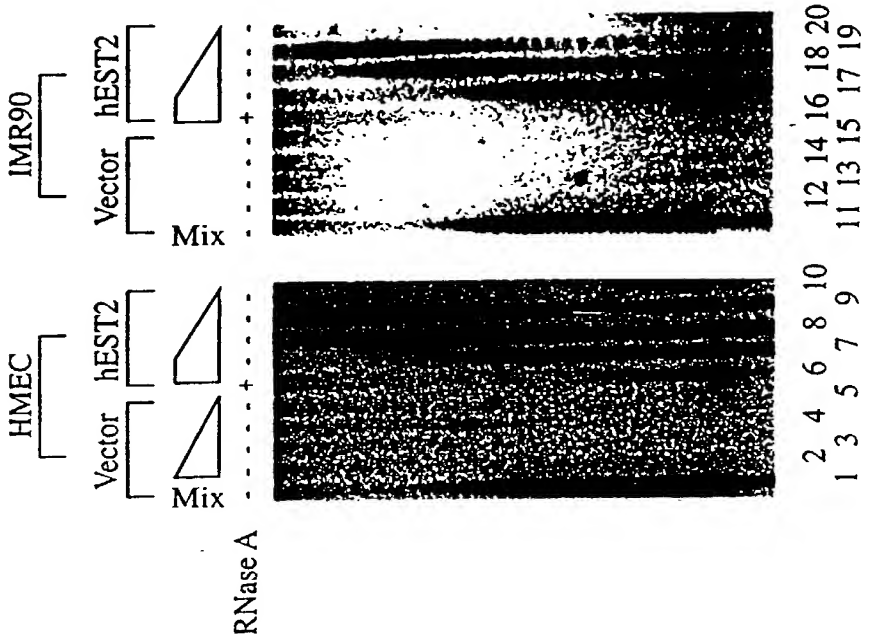

Fig. 6B

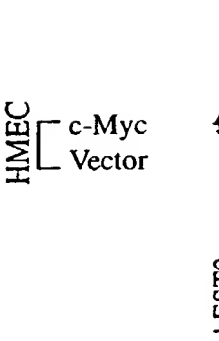

Fig. 6A

Fig. 6C

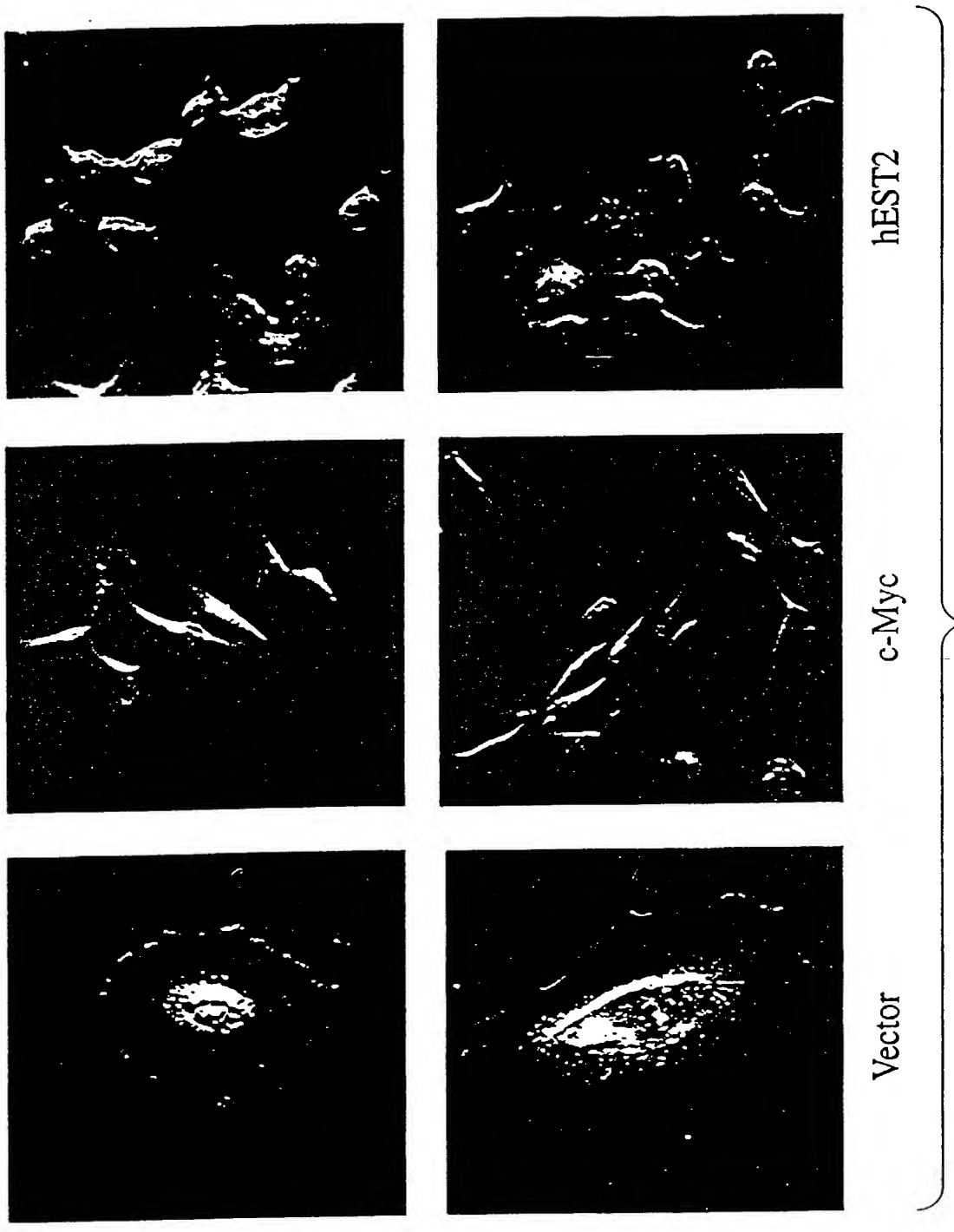

Fig. 6D

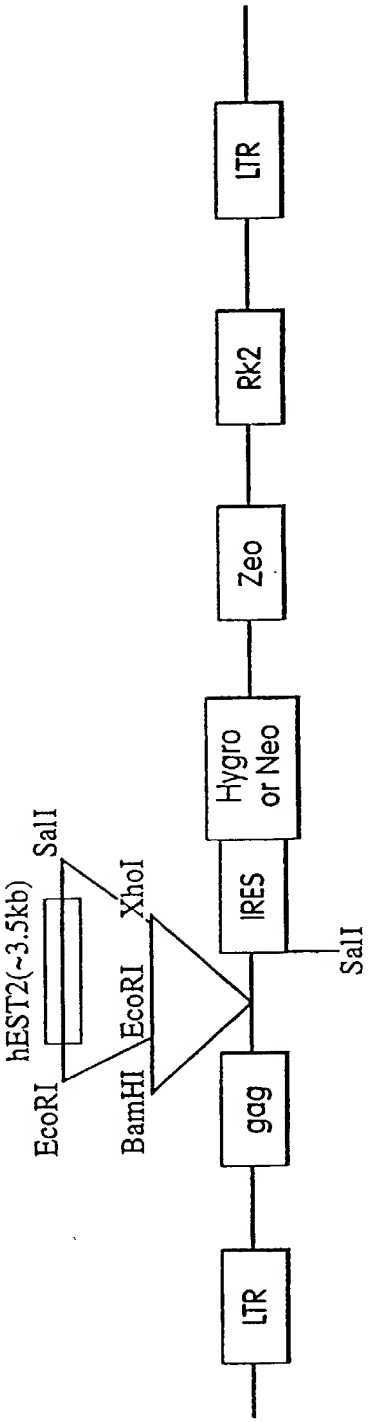
LTR
Rk2
Zeo
Hygro or Neo
IRES
SalI
gag
LTR
SalI
XhoI
EcoRI
BamHI
hEST2(~3.5kb)
EcoRI

Fig. 7

Docket Number GNCA-P01-005

DECLARATION FOR UTILITY PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS AND REAGENTS FOR INCREASING PROLIFERATIVE CAPACITY AND PREVENTING REPLICATIVE SENESCENCE

the specification of which (check one)

☐ is attached hereto
☒ was filed on September 7, 2001 as United States Application Number 09/936,035, and on November 24, 1999 as PCT/US99/27907. I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose information, which is material to patentability as defined in Title 37, Code of Federal Regulation, § 1 56

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed

Prior Foreign Application(s)			Priority Claimed
PCT/US99/27907 (Number)	International (Country)	24 November 1999 (Day/Month/Year Filed)	☒ Yes ☐ No

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States Provisional application(s) listed below.

60/109,891 (Application Number)	25 November 1998 (Filing Date)
60/120,549 (Application Number)	17 February 1999 (Filing Date)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number)	(Filing Date)	(Status: patented, pending, abandoned)

I hereby appoint Madeline Baer, Reg. No. 36,437; Steven J. Baughman, Reg. No P-47,414; Johnny Y. Chen, Reg. No. 46,614; Gregory G Glover, Reg No. 34,173; Patricia Granahan, Reg. No. 32,227; David P. Halstead, Reg No. 44,735, Daniel Hansburg, Reg No. 36,156; Edward J. Kelly, Reg. No. 38,936; Robert A. Mazzarese, Reg. No. 42,852; Colleen H. McDuffie, Reg. No 43,788; Robert H. Morse, Reg. No 25,358; James L Sigel, Reg. No. 39,029; Wolfgang Stutius, Reg. No. 40,256; Matthew P. Vincent, Reg. No. 36,709; as attorneys/agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all telephone calls to Matthew P. Vincent at telephone number (617) 951-7000.

Address all correspondence to: **Customer Id No: 28120**

Patent Group
Ropes & Gray LLP
One International Place
Boston, Ma. 02110-2624

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name): **Gregory J. Hannon**
Inventor's signature: _____ Date: _____
Residence: 34 Griffith Lane, Huntington, New York 11743 Citizenship: United States of America
Post Office Address: Same as above

200

Full name of second inventor (given name, family name): **David H. Beach**
Inventor's signature: D.H. Beach Date: 04/04/2002
Residence: 492 Beacon Street, No 11, Boston, Massachusetts 02115 Citizenship: Great Britain
Post Office Address: Same as above MH

Docket Number GNCA-P01-005

DECLARATION FOR UTILITY PATENT APPLICATION

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHODS AND REAGENTS FOR INCREASING PROLIFERATIVE CAPACITY AND PREVENTING REPLICATIVE SENESCENCE

the specification of which (check one)

☐ is attached hereto.
☒ was filed on September 7, 2001 as United States Application Number 09/936,035, and on November 24, 1999 as PCT/US99/27907.
I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed
PCT/US99/27907 (Number)	International (Country)	24 November 1999 (Day/Month/Year Filed)	☒ Yes ☐ No

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States Provisional application(s) listed below.

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(Application Number)	(Filing Date)	(Status: patented, pending, abandoned)

I hereby appoint Madeline Baer, Reg. No. 36,437; Steven J. Baughman, Reg. No. P-47,414; Johnny Y. Chen, Reg. No. 46,614; Gregory G. Glover, Reg. No. 34,173; Patricia Granahan, Reg. No. 32,227; David P. Halstead, Reg. No. 44,735; Daniel Hansburg, Reg. No. 36,156; Edward J. Kelly, Reg. No. 38,936; Robert A. Mazzarese, Reg. No. 42,852; Colleen H. McDuffie, Reg. No. 43,788; Robert H. Morse, Reg. No. 25,358; James L. Sigel, Reg. No. 39,029; Wolfgang Stutius, Reg. No. 40,256; Matthew P. Vincent, Reg. No. 36,709; as attorneys/agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

100

Full name of sole or first inventor (given name, ~~family name~~): **Gregory J. Hannon**
Inventor's signature: _____ Date: 4/3/02
Residence: 34 Griffith Lane, Huntington, New York 11743 Citizenship: United States of America
Post Office Address: Same as above N/A

Full name of second inventor (given name, family name): **David H. Beach**
Inventor's signature: _____ Date: _____
Residence: 492 Beacon Street, No. 11, Boston, Massachusetts 02115 Citizenship: Great Britain
Post Office Address: Same as above

Sequence Listing

SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4027 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 57..3452

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Pro Arg Ala Pro Arg Cys Arg Ala Val Arg Ser Leu Leu Arg Ser
 1               5                  10                  15

CAC TAC CGC GAG GTG CTG CCG CTG GCC ACG TTC GTG CGG CGC CTG GGG       152
His Tyr Arg Glu Val Leu Pro Leu Ala Thr Phe Val Arg Arg Leu Gly
            20                  25                  30

CCC CAG GGC TGG CGG CTG GTG CAG CGC GGG GAC CCG GCG GCT TTC CGC       200
Pro Gln Gly Trp Arg Leu Val Gln Arg Gly Asp Pro Ala Ala Phe Arg
        35                  40                  45

GCG CTG GTG GCC CAG TGC CTG GTG TGC GTG CCC TGG GAC GCA CGG CCG       248
Ala Leu Val Ala Gln Cys Leu Val Cys Val Pro Trp Asp Ala Arg Pro
    50                  55                  60

CCC CCC GCC GCC CCC TCC TTC CGC CAG GTG TCC TGC CTG AAG GAG CTG       296
Pro Pro Ala Ala Pro Ser Phe Arg Gln Val Ser Cys Leu Lys Glu Leu
65                  70                  75                  80

GTG GCC CGA GTG CTG CAG AGG CTG TGC GAG CGC GGC GCG AAG AAC GTG       344
Val Ala Arg Val Leu Gln Arg Leu Cys Glu Arg Gly Ala Lys Asn Val
                85                  90                  95

CTG GCC TTC GGC TTC GCG CTG CTG GAC GGG GCC CGC GGG GGC CCC CCC       392
Leu Ala Phe Gly Phe Ala Leu Leu Asp Gly Ala Arg Gly Gly Pro Pro
            100                 105                 110

GAG GCC TTC ACC ACC AGC GTG CGC AGC TAC CTG CCC AAC ACG GTG ACC       440
Glu Ala Phe Thr Thr Ser Val Arg Ser Tyr Leu Pro Asn Thr Val Thr
        115                 120                 125

GAC GCA CTG CGG GGG AGC GGG GCG TGG GGG CTG CTG TTG CGC CGC GTG       488
Asp Ala Leu Arg Gly Ser Gly Ala Trp Gly Leu Leu Leu Arg Arg Val
    130                 135                 140

GGC GAC GAC GTG CTG GTT CAC CTG CTG GCA CGC TGC GCG CTC TTT GTG       536
Gly Asp Asp Val Leu Val His Leu Leu Ala Arg Cys Ala Leu Phe Val
145                 150                 155                 160

CTG GTG GCT CCC AGC TGC GCC TAC CAG GTG TGC GGG CCG CCG CTG TAC       584
Leu Val Ala Pro Ser Cys Ala Tyr Gln Val Cys Gly Pro Pro Leu Tyr
                165                 170                 175

CAG CTC GGC GCT GCC ACT CAG GCC CGG CCC CCG CCA CAC GCT AGT GGA       632
Gln Leu Gly Ala Ala Thr Gln Ala Arg Pro Pro Pro His Ala Ser Gly
            180                 185                 190

CCC CGA AGG CGT CTG GGA TGC GAA CGG GCC TGG AAC CAT AGC GTC AGG       680
Pro Arg Arg Arg Leu Gly Cys Glu Arg Ala Trp Asn His Ser Val Arg
        195                 200                 205
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Glu Ala Gly Val Pro Leu Gly Leu Pro Ala Pro Gly Ala Arg Arg Arg
    210                 215                 220

GGG GGC AGT GCC AGC CGA AGT CTG CCG TTG CCC AAG AGG CCC AGG CGT        776
Gly Gly Ser Ala Ser Arg Ser Leu Pro Leu Pro Lys Arg Pro Arg Arg
225                 230                 235                 240

GGC GCT GCC CCT GAG CCG GAG CGG ACG CCC GTT GGG CAG GGG TCC TGG        824
Gly Ala Ala Pro Glu Pro Glu Arg Thr Pro Val Gly Gln Gly Ser Trp
                245                 250                 255

GCC CAC CCG GGC AGG ACG CGT GGA CCG AGT GAC CGT GGT TTC TGT GTG        872
Ala His Pro Gly Arg Thr Arg Gly Pro Ser Asp Arg Gly Phe Cys Val
            260                 265                 270

GTG TCA CCT GCC AGA CCC GCC GAA GAA GCC ACC TCT TTG GAG GGT GCG        920
Val Ser Pro Ala Arg Pro Ala Glu Glu Ala Thr Ser Leu Glu Gly Ala
        275                 280                 285

CTC TCT GGC ACG CGC CAC TCC CAC CCA TCC GTG GGC CGC CAG CAC CAC        968
Leu Ser Gly Thr Arg His Ser His Pro Ser Val Gly Arg Gln His His
    290                 295                 300

GCG GGC CCC CCA TCC ACA TCG CGG CCA CCA CGT CCC TGG GAC ACG CCT       1016
Ala Gly Pro Pro Ser Thr Ser Arg Pro Pro Arg Pro Trp Asp Thr Pro
305                 310                 315                 320

TGT CCC CCG GTG TAC GCC GAG ACC AAG CAC TTC CTC TAC TCC TCA GGC       1064
Cys Pro Pro Val Tyr Ala Glu Thr Lys His Phe Leu Tyr Ser Ser Gly
                325                 330                 335

GAC AAG GAG CAG CTG CGG CCC TCC TTC CTA CTC AGC TCT CTG AGG CCC       1112
Asp Lys Glu Gln Leu Arg Pro Ser Phe Leu Leu Ser Ser Leu Arg Pro
            340                 345                 350

AGC CTG ACT GGC GCT CGG AGG CTC GTG GAG ACC ATC TTT CTG GGT TCC       1160
Ser Leu Thr Gly Ala Arg Arg Leu Val Glu Thr Ile Phe Leu Gly Ser
        355                 360                 365

AGG CCC TGG ATG CCA GGG ACT CCC CGC AGG TTG CCC CGC CTG CCC CAG       1208
Arg Pro Trp Met Pro Gly Thr Pro Arg Arg Leu Pro Arg Leu Pro Gln
    370                 375                 380

CGC TAC TGG CAA ATG CGG CCC CTG TTT CTG GAG CTG CTT GGG AAC CAC       1256
Arg Tyr Trp Gln Met Arg Pro Leu Phe Leu Glu Leu Leu Gly Asn His
385                 390                 395                 400

GCG CAG TGC CCC TAC GGG GTG CTC CTC AAG ACG CAC TGC CCG CTG CGA       1304
Ala Gln Cys Pro Tyr Gly Val Leu Leu Lys Thr His Cys Pro Leu Arg
                405                 410                 415

GCT GCG GTC ACC CCA GCA GCC GGT GTC TGT GCC CGG GAG AAG CCC CAG       1352
Ala Ala Val Thr Pro Ala Ala Gly Val Cys Ala Arg Glu Lys Pro Gln
            420                 425                 430

GGC TCT GTG GCG GCC CCC GAG GAG GAG GAC ACA GAC CCC CGT CGC CTG       1400
Gly Ser Val Ala Ala Pro Glu Glu Glu Asp Thr Asp Pro Arg Arg Leu
        435                 440                 445

GTG CAG CTG CTC CGC CAG CAC AGC AGC CCC TGG CAG GTG TAC GGC TTC       1448
Val Gln Leu Leu Arg Gln His Ser Ser Pro Trp Gln Val Tyr Gly Phe
    450                 455                 460

GTG CGG GCC TGC CTG CGC CGG CTG GTG CCC CCA GGC CTC TGG GGC TCC       1496
Val Arg Ala Cys Leu Arg Arg Leu Val Pro Pro Gly Leu Trp Gly Ser
465                 470                 475                 480

AGG CAC AAC GAA CGC CGC TTC CTC AGG AAC ACC AAG AAG TTC ATC TCC       1544
Arg His Asn Glu Arg Arg Phe Leu Arg Asn Thr Lys Lys Phe Ile Ser
                485                 490                 495

CTG GGG AAG CAT GCC AAG CTC TCG CTG CAG GAG CTG ACG TGG AAG ATG       1592
Leu Gly Lys His Ala Lys Leu Ser Leu Gln Glu Leu Thr Trp Lys Met
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AGC GTG CGG GGC TGC GCT TGG CTG CGC AGG AGC CCA GGG GTT GGC TGT      1640
Ser Val Arg Gly Cys Ala Trp Leu Arg Arg Ser Pro Gly Val Gly Cys
        515                 520                 525

GTT CCG GCC GCA GAG CAC CGT CTG CGT GAG GAG ATC CTG GCC AAG TTC      1688
Val Pro Ala Ala Glu His Arg Leu Arg Glu Glu Ile Leu Ala Lys Phe
    530                 535                 540

CTG CAC TGG CTG ATG AGT GTG TAC GTC GTC GAG CTG CTC AGG TCT TTC      1736
Leu His Trp Leu Met Ser Val Tyr Val Val Glu Leu Leu Arg Ser Phe
545                 550                 555                 560

TTT TAT GTC ACG GAG ACC ACG TTT CAA AAG AAC AGG CTC TTT TTC TAC      1784
Phe Tyr Val Thr Glu Thr Thr Phe Gln Lys Asn Arg Leu Phe Phe Tyr
                565                 570                 575

CGG AAG AGT GTC TGG AGC AAG TTG CAA AGC ATT GGA ATC AGA CAG CAC      1832
Arg Lys Ser Val Trp Ser Lys Leu Gln Ser Ile Gly Ile Arg Gln His
            580                 585                 590

TTG AAG AGG GTG CAG CTG CGG GAG CTG TCG GAA GCA GAG GTC AGG CAG      1880
Leu Lys Arg Val Gln Leu Arg Glu Leu Ser Glu Ala Glu Val Arg Gln
        595                 600                 605

CAT CGG GAA GCC AGG CCC GCC CTG CTG ACG TCC AGA CTC CGC TTC ATC      1928
His Arg Glu Ala Arg Pro Ala Leu Leu Thr Ser Arg Leu Arg Phe Ile
    610                 615                 620

CCC AAG CCT GAC GGG CTG CGG CCG ATT GTG AAC ATG GAC TAC GTC GTG      1976
Pro Lys Pro Asp Gly Leu Arg Pro Ile Val Asn Met Asp Tyr Val Val
625                 630                 635                 640

GGA GCC AGA ACG TTC CGC AGA GAA AAG AGG GCC GAG CGT CTC ACC TCG      2024
Gly Ala Arg Thr Phe Arg Arg Glu Lys Arg Ala Glu Arg Leu Thr Ser
                645                 650                 655

AGG GTG AAG GCA CTG TTC AGC GTG CTC AAC TAC GAG CGG GCG CGG CGC      2072
Arg Val Lys Ala Leu Phe Ser Val Leu Asn Tyr Glu Arg Ala Arg Arg
            660                 665                 670

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Pro Gly Leu Leu Gly Ala Ser Val Leu Gly Leu Asp Asp Ile His Arg
        675                 680                 685

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Ala Trp Arg Thr Phe Val Leu Arg Val Arg Ala Gln Asp Pro Pro Pro
    690                 695                 700

GAG CTG TAC TTT GTC AAG GTG GAT GTG ACG GGC GCG TAC GAC ACC ATC      2216
Glu Leu Tyr Phe Val Lys Val Asp Val Thr Gly Ala Tyr Asp Thr Ile
705                 710                 715                 720

CCC CAG GAC AGG CTC ACG GAG GTC ATC GCC AGC ATC ATC AAA CCC CAG      2264
Pro Gln Asp Arg Leu Thr Glu Val Ile Ala Ser Ile Ile Lys Pro Gln
                725                 730                 735

AAC ACG TAC TGC GTG CGT CGG TAT GCC GTG GTC CAG AAG GCC GCC CAT      2312
Asn Thr Tyr Cys Val Arg Arg Tyr Ala Val Val Gln Lys Ala Ala His
            740                 745                 750

GGG CAC GTC CGC AAG GCC TTC AAG AGC CAC GTC TCT ACC TTG ACA GAC      2360
Gly His Val Arg Lys Ala Phe Lys Ser His Val Ser Thr Leu Thr Asp
        755                 760                 765

CTC CAG CCG TAC ATG CGA CAG TTC GTG GCT CAC CTG CAG GAG ACC AGC      2408
Leu Gln Pro Tyr Met Arg Gln Phe Val Ala His Leu Gln Glu Thr Ser
    770                 775                 780

CCG CTG AGG GAT GCC GTC GTC ATC GAG CAG AGC TCC TCC CTG AAT GAG      2456
Pro Leu Arg Asp Ala Val Val Ile Glu Gln Ser Ser Ser Leu Asn Glu
785                 790                 795                 800

GCC AGC AGT GGC CTC TTC GAC GTC TTC CTA CGC TTC ATG TGC CAC CAC      2504
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Ala Ser Ser Gly Leu Phe Asp Val Phe Leu Arg Phe Met Cys His His
805                             810                             815

GCC GTG CGC ATC AGG GGC AAG TCC TAC GTC CAG TGC CAG GGG ATC CCG      2552
Ala Val Arg Ile Arg Gly Lys Ser Tyr Val Gln Cys Gln Gly Ile Pro
            820                 825                 830

CAG GGC TCC ATC CTC TCC ACG CTG CTC TGC AGC CTG TGC TAC GGC GAC      2600
Gln Gly Ser Ile Leu Ser Thr Leu Leu Cys Ser Leu Cys Tyr Gly Asp
        835                 840                 845

ATG GAG AAC AAG CTG TTT GCG GGG ATT CGG CGG GAC GGG CTG CTC CTG      2648
Met Glu Asn Lys Leu Phe Ala Gly Ile Arg Arg Asp Gly Leu Leu Leu
    850                 855                 860

CGT TTG GTG GAT GAT TTC TTG TTG GTG ACA CCT CAC CTC ACC CAC GCG      2696
Arg Leu Val Asp Asp Phe Leu Leu Val Thr Pro His Leu Thr His Ala
865                 870                 875                 880

AAA ACC TTC CTC AGG ACC CTG GTC CGA GGT GTC CCT GAG TAT GGC TGC      2744
Lys Thr Phe Leu Arg Thr Leu Val Arg Gly Val Pro Glu Tyr Gly Cys
                885                 890                 895

GTG GTG AAC TTG CGG AAG ACA GTG GTG AAC TTC CCT GTA GAA GAC GAG      2792
Val Val Asn Leu Arg Lys Thr Val Val Asn Phe Pro Val Glu Asp Glu
            900                 905                 910

GCC CTG GGT GGC ACG GCT TTT GTT CAG ATG CCG GCC CAC GGC CTA TTC      2840
Ala Leu Gly Gly Thr Ala Phe Val Gln Met Pro Ala His Gly Leu Phe
        915                 920                 925

CCC TGG TGC GGC CTG CTG CTG GAT ACC CGG ACC CTG GAG GTG CAG AGC      2888
Pro Trp Cys Gly Leu Leu Leu Asp Thr Arg Thr Leu Glu Val Gln Ser
    930                 935                 940

GAC TAC TCC AGC TAT GCC CGG ACC TCC ATC AGA GCC AGT CTC ACC TTC      2936
Asp Tyr Ser Ser Tyr Ala Arg Thr Ser Ile Arg Ala Ser Leu Thr Phe
945                 950                 955                 960

AAC CGC GGC TTC AAG GCT GGG AGG AAC ATG CGT CGC AAA CTC TTT GGG      2984
Asn Arg Gly Phe Lys Ala Gly Arg Asn Met Arg Arg Lys Leu Phe Gly
                965                 970                 975

GTC TTG CGG CTG AAG TGT CAC AGC CTG TTT CTG GAT TTG CAG GTG AAC      3032
Val Leu Arg Leu Lys Cys His Ser Leu Phe Leu Asp Leu Gln Val Asn
            980                 985                 990

AGC CTC CAG ACG GTG TGC ACC AAC ATC TAC AAG ATC CTC CTG CTG CAG      3080
Ser Leu Gln Thr Val Cys Thr Asn Ile Tyr Lys Ile Leu Leu Leu Gln
        995                 1000                1005

GCG TAC AGG TTT CAC GCA TGT GTG CTG CAG CTC CCA TTT CAT CAG CAA      3128
Ala Tyr Arg Phe His Ala Cys Val Leu Gln Leu Pro Phe His Gln Gln
    1010                1015                1020

GTT TGG AAG AAC CCC ACA TTT TTC CTG CGC GTC ATC TCT GAC ACG GCC      3176
Val Trp Lys Asn Pro Thr Phe Phe Leu Arg Val Ile Ser Asp Thr Ala
1025                1030                1035                1040

TCC CTC TGC TAC TCC ATC CTG AAA GCC AAG AAC GCA GGG ATG TCG CTG      3224
Ser Leu Cys Tyr Ser Ile Leu Lys Ala Lys Asn Ala Gly Met Ser Leu
                1045                1050                1055

GGG GCC AAG GGC GCC GCC GGC CCT CTG CCC TCC GAG GCC GTG CAG TGG      3272
Gly Ala Lys Gly Ala Ala Gly Pro Leu Pro Ser Glu Ala Val Gln Trp
            1060                1065                1070

CTG TGC CAC CAA GCA TTC CTG CTC AAG CTG ACT CGA CAC CGT GTC ACC      3320
Leu Cys His Gln Ala Phe Leu Leu Lys Leu Thr Arg His Arg Val Thr
        1075                1080                1085

TAC GTG CCA CTC CTG GGG TCA CTC AGG ACA GCC CAG ACG CAG CTG AGT      3368
Tyr Val Pro Leu Leu Gly Ser Leu Arg Thr Ala Gln Thr Gln Leu Ser
    1090                1095                1100
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CCG GCA CTG CCC TCA GAC TTC AAG ACC ATC CTG GAC TGATGGCCAC          3462
Pro Ala Leu Pro Ser Asp Phe Lys Thr Ile Leu Asp
                    1125                1130

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TGGAGTGACC AAAGGTGTGC CCTGTACACA GGCGAGGACC CTGCACCTGG ATGGGGGTCC   3942

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AGTTTTGAAA AAAAAAAAAA AAAAA                                         4027
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SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1132 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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His Tyr Arg Glu Val Leu Pro Leu Ala Thr Phe Val Arg Arg Leu Gly
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Pro Gln Gly Trp Arg Leu Val Gln Arg Gly Asp Pro Ala Ala Phe Arg
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Ala Leu Val Ala Gln Cys Leu Val Cys Val Pro Trp Asp Ala Arg Pro
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Pro Pro Ala Ala Pro Ser Phe Arg Gln Val Ser Cys Leu Lys Glu Leu
65                  70                  75                  80

Val Ala Arg Val Leu Gln Arg Leu Cys Glu Arg Gly Ala Lys Asn Val
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Leu Ala Phe Gly Phe Ala Leu Leu Asp Gly Ala Arg Gly Gly Pro Pro
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Glu Ala Phe Thr Thr Ser Val Arg Ser Tyr Leu Pro Asn Thr Val Thr
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Asp Ala Leu Arg Gly Ser Gly Ala Trp Gly Leu Leu Leu Arg Arg Val
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Gly Asp Asp Val Leu Val His Leu Leu Ala Arg Cys Ala Leu Phe Val
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Gln Leu Gly Ala Ala Thr Gln Ala Arg Pro Pro Pro His Ala Ser Gly
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Pro Arg Arg Arg Leu Gly Cys Glu Arg Ala Trp Asn His Ser Val Arg
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Gly Gly Ser Ala Ser Arg Ser Leu Pro Leu Pro Lys Arg Pro Arg Arg
225 230 235 240

Gly Ala Ala Pro Glu Pro Glu Arg Thr Pro Val Gly Gln Gly Ser Trp
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Ala His Pro Gly Arg Thr Arg Gly Pro Ser Asp Arg Gly Phe Cys Val
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Val Ser Pro Ala Arg Pro Ala Glu Glu Ala Thr Ser Leu Glu Gly Ala
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Leu Ser Gly Thr Arg His Ser His Pro Ser Val Gly Arg Gln His His
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Ala Gly Pro Pro Ser Thr Ser Arg Pro Pro Arg Pro Trp Asp Thr Pro
305 310 315 320

Cys Pro Pro Val Tyr Ala Glu Thr Lys His Phe Leu Tyr Ser Ser Gly
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Asp Lys Glu Gln Leu Arg Pro Ser Phe Leu Leu Ser Ser Leu Arg Pro
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Arg Pro Trp Met Pro Gly Thr Pro Arg Arg Leu Pro Arg Leu Pro Gln
370 375 380

Arg Tyr Trp Gln Met Arg Pro Leu Phe Leu Glu Leu Leu Gly Asn His
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405 410 415

Ala Ala Val Thr Pro Ala Ala Gly Val Cys Ala Arg Glu Lys Pro Gln
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Gly Ser Val Ala Ala Pro Glu Glu Glu Asp Thr Asp Pro Arg Arg Leu
435 440 445

Val Gln Leu Leu Arg Gln His Ser Ser Pro Trp Gln Val Tyr Gly Phe
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Val Arg Ala Cys Leu Arg Arg Leu Val Pro Pro Gly Leu Trp Gly Ser
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Arg His Asn Glu Arg Arg Phe Leu Arg Asn Thr Lys Lys Phe Ile Ser
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515 520 525

Val Pro Ala Ala Glu His Arg Leu Arg Glu Glu Ile Leu Ala Lys Phe
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Leu His Trp Leu Met Ser Val Tyr Val Val Glu Leu Leu Arg Ser Phe
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Phe Tyr Val Thr Glu Thr Thr Phe Gln Lys Asn Arg Leu Phe Phe Tyr
565 570 575

Arg Lys Ser Val Trp Ser Lys Leu Gln Ser Ile Gly Ile Arg Gln His

580 585 590

Leu Lys Arg Val Gln Leu Arg Glu Leu Ser Glu Ala Glu Val Arg Gln
595 600 605

His Arg Glu Ala Arg Pro Ala Leu Leu Thr Ser Arg Leu Arg Phe Ile
610 615 620

Pro Lys Pro Asp Gly Leu Arg Pro Ile Val Asn Met Asp Tyr Val Val
625 630 635 640

Gly Ala Arg Thr Phe Arg Arg Glu Lys Arg Ala Glu Arg Leu Thr Ser
645 650 655

Arg Val Lys Ala Leu Phe Ser Val Leu Asn Tyr Glu Arg Ala Arg Arg
660 665 670

Pro Gly Leu Leu Gly Ala Ser Val Leu Gly Leu Asp Asp Ile His Arg
675 680 685

Ala Trp Arg Thr Phe Val Leu Arg Val Arg Ala Gln Asp Pro Pro Pro
690 695 700

Glu Leu Tyr Phe Val Lys Val Asp Val Thr Gly Ala Tyr Asp Thr Ile
705 710 715 720

Pro Gln Asp Arg Leu Thr Glu Val Ile Ala Ser Ile Ile Lys Pro Gln
725 730 735

Asn Thr Tyr Cys Val Arg Arg Tyr Ala Val Val Gln Lys Ala Ala His
740 745 750

Gly His Val Arg Lys Ala Phe Lys Ser His Val Ser Thr Leu Thr Asp
755 760 765

Leu Gln Pro Tyr Met Arg Gln Phe Val Ala His Leu Gln Glu Thr Ser
770 775 780

Pro Leu Arg Asp Ala Val Val Ile Glu Gln Ser Ser Ser Leu Asn Glu
785 790 795 800

Ala Ser Ser Gly Leu Phe Asp Val Phe Leu Arg Phe Met Cys His His
805 810 815

Ala Val Arg Ile Arg Gly Lys Ser Tyr Val Gln Cys Gln Gly Ile Pro
820 825 830

Gln Gly Ser Ile Leu Ser Thr Leu Leu Cys Ser Leu Cys Tyr Gly Asp
835 840 845

Met Glu Asn Lys Leu Phe Ala Gly Ile Arg Arg Asp Gly Leu Leu Leu
850 855 860

Arg Leu Val Asp Asp Phe Leu Leu Val Thr Pro His Leu Thr His Ala
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Lys Thr Phe Leu Arg Thr Leu Val Arg Gly Val Pro Glu Tyr Gly Cys
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Val Val Asn Leu Arg Lys Thr Val Val Asn Phe Pro Val Glu Asp Glu
900 905 910

Ala Leu Gly Gly Thr Ala Phe Val Gln Met Pro Ala His Gly Leu Phe
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Pro Trp Cys Gly Leu Leu Leu Asp Thr Arg Thr Leu Glu Val Gln Ser
930 935 940

Asp Tyr Ser Ser Tyr Ala Arg Thr Ser Ile Arg Ala Ser Leu Thr Phe
945 950 955 960

Asn Arg Gly Phe Lys Ala Gly Arg Asn Met Arg Arg Lys Leu Phe Gly
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Val Leu Arg Leu Lys Cys His Ser Leu Phe Leu Asp Leu Gln Val Asn

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Ser Leu Gln Thr Val Cys Thr Asn Ile Tyr Lys Ile Leu Leu Leu Gln
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Ala Tyr Arg Phe His Ala Cys Val Leu Gln Leu Pro Phe His Gln Gln
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Val Trp Lys Asn Pro Thr Phe Phe Leu Arg Val Ile Ser Asp Thr Ala
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Ser Leu Cys Tyr Ser Ile Leu Lys Ala Lys Asn Ala Gly Met Ser Leu
                1045                1050                1055

Gly Ala Lys Gly Ala Ala Gly Pro Leu Pro Ser Glu Ala Val Gln Trp
            1060                1065                1070

Leu Cys His Gln Ala Phe Leu Leu Lys Leu Thr Arg His Arg Val Thr
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Tyr Val Pro Leu Leu Gly Ser Leu Arg Thr Ala Gln Thr Gln Leu Ser
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Arg Lys Leu Pro Gly Thr Thr Leu Thr Ala Leu Glu Ala Ala Ala Asn
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Pro Ala Leu Pro Ser Asp Phe Lys Thr Ile Leu Asp
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SEQ ID NO: 3

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	/organism="Homo sapiens"
	/db_xref="taxon:9606"
gene	312..1787
	/gene="p53 associated"
CDS	312..1787
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	/db_xref="PID:g35212"
	/db_xref="SWISS-PROT:Q00987"

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 121 cagccaggag caccgtccct ccccggatta gtgcgtacga gcgcccagtg ccctggcccg
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 241 aaggaaactg gggagtcttg agggaccccc gactccaagc gcgaaaaccc cggatggtga
 301 ggagcaggca aatgtgcaat accaacatgt ctgtacctac tgatggtgct gtaaccacct
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 661 actcaggtac atctgtgagt gagaacaggt gtcaccttga aggtgggagt gatcaaaagg
 721 accttgtaca agagcttcag gaagagaaac cttcatcttc acatttggtt tctagaccat
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 841 gtgaacgaca aagaaaacgc cacaaatctg atagtatttc cctttccttt gatgaaagcc
 901 tggctctgtg tgtaataagg gagatatgtt gtgaaagaag cagtagcagt gaatctacag
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SEQ ID No: 4

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SEQ ID No. 5

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	2949..2999,3135..3270,3836..4233)
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exon	85..291
	/gene="CDK4"
gene	85..4233
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	/db_xref="PID:g1353416"
exon	1123..1258
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exon	1376..1543
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BASE COUNT 945 a 1050 c 1101 g 1124 t 13 others

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SEQ ID No. 6

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301 npe
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SEQ ID No. 7

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SEQ ID No. 8

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SEQ ID No. 9

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SEQ ID No. 10

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SEQ ID No. 11

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 540 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 142..540
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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☐ Page(s)_____ of _____ were not present for scanning. (Document title)

There are only 11 pages of drawings NOt 12.

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